"STABILITY INDICATING RP-HPLC ESTIMATION OF DROTAVERINE HYDROCHLORIDE IN PHARMACEUTICAL FORMULATIONS"

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In

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BY

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DECLARATION

*I declare that the thesis entitled "**STABILITY INDICATING RP-HPLC ESTIMATION OF DROTAVERINE HYDROCHLORIDE IN PHARMACEUTICAL FORMULATIONS**" has been prepared by me under the guidance of Dr. PRITI J. MEHTA, Senior Associate Professor, Department of Pharmaceutical Analysis, Nirma University. No part of this thesis has formed the basis for the award of any degree or fellowship previously.*

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Date: ***Sagar D. Shah***

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ABBREVIATIONS

CHEMICALS:

DRT HCl	Drotaverine Hydrochloride
ACN	Acetonitrile
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric acid
NaOH	Sodium Hydroxide
MeOH	Methanol
gl HAC	Glacial Acetic Acid
TEA	Tri Ethyl Amine
H_2O	Water
KH_2PO_4	Potassium Di Hydrogen Phophate

SYMBOL:

%	Percentage
Rs	Resolution
k’	Capacity Factor
α	Selectivity
N	Column Efficiency
As	Peak Asymmetric Factor
T	Tailing Factor
λ_{max}	Wavelength of maximum absorption
<	Less than
>	Greater than
µg	Microgram (S)
µL	Micro liter (S)
µm	Micro meter (S)
cm	Centimeter (S)
i.d.	Internal diameter
L	Liter (S)
M	Molar
mg	Milligram (S)

Min	Minute (S)
mL	Milliliter (S)
mM	Milimolar
mm	Millimeter (S)
nm	Nanometer (S)
r^2	Correlation coefficient
Sec	Second (S)
Temp	Temperature
°C	Degree centigrade
No.	Number
Sr.No.	Serial number
N	Normal
v/v	Volume by volume
w/w	Weight by weight
Conc.	Concentration
ppm	Parts per million

OTHERS:

SIAM	Stability Indicating Assay Method
API	Active Pharmaceutical Ingredient
RSD	Relative Standard Deviation
STD	Standard Deviation
USP	United States Pharmacopoeia
ICH	International Conference On Harmonization
FDA	Food & Drug Adminstration
GMP	Good Manufacturing Practice
DS	Drug Substance
DP	DRUG PRODUCT
UV	Ultraviolet
IR	Infrared
TLC	Thin Layer Chromatography
HPTLC	High Performance Thin Layer Chromatography
HPLC	High Performance Liquid Chromatography
GC	Gas Chromatography

MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
GC-MS	Gas Chromatography- Mass Spectroscopy
LC-MS	Liquid Chromatography -Mass Spectroscopy
LC-NMR	Liquid Chromatography- Nuclear Magnetic Resonance
CE	Capillary Electrophoresis
PDA	Photo Diode Array
PDE	Phosphodiesterase Enzymes
FR	Flow Rate
RI	Refractive Index
ELSD	Evaporating Light Scattering Detector
AOAC	American Association of Official Analytical Chemists
IUPAC	International Union of Pure And Applied Chemistry
pKa	Weak Acid Ionization Constant
SSP	System Suitability Parameters
Hrs	Hours
RT	Retention Time
MP	Mobile Phase
RRF	Relative response factor
RRT	Relative Retention Time
LOD	Limit of detection
LOQ	Limit of Quantitation
RBF	Round Bottom Flask
ODS	Octa Decyl Silane
ISE	Ion Selective Electrode
PVC	Poly Venyl Chloride

Drugs are an inevitable part of our life. Quality of the drug is an essential feature as it directly affects the life of the consumer. Quality of any product or material can be judged by analyzing it, only. Science provides truth through its investigative process called analysis. Analytical chemistry is the science of obtaining, processing and communicating information about the composition and structure of matter. The number of the drugs introduced in the market is increasing every day.

These drugs are either the new entities or the structural modification of the existing drugs. Very often there is time lag from the date of introduction of a drug in to market to the date of its inclusion in pharmacopoeia. Under these conditions, standard and analytical process for these drugs may not be available in the pharmacopoeia. It becomes necessary to develop new analytical methods for such drugs.

The reasons for the development of newer analytical methods of drugs are:

- The drug / drug combination may not be official in any pharmacopoeia.
- Proper analytical procedure for estimation of drug may not be available in literature due to patent regulations.
- Analytical methods for estimation of the drugs in the formulations may not be available.
- Analytical methods for the quantification in the biological fluids may not be available.
- Existing analytical method may require expensive reagent or solvent.
- Impurity profiling of drugs may not be available.

1.1. INTRODUCTION TO STABILITY INDICATING ASSAY METHOD(SIAM)

- **Stability:**

Stability is a critical quality attribute of the pharmaceutical product therefore stability testing plays a crucial role in the drug development process. [(1)]

- **SIAM[(2)]:**

According to regulatory definition, a SIAM is one of a number of quantitative analytical methods that are based on characteristics structural, chemical or biological properties of each active ingredient of a Drug Product and that will distinguish each API from its degradants so that API content can be accurately measured.

So, SIAM is an analytical procedure that is capable of discriminating between major API from any degradation products formed under defined storage condition during stability evaluation period.

Purpose:

- To provide evidences on how quality of drug substance or product varies with time under the influences of variety of environmental conditions such as temperature, pH, humidity and light.
- To establish:
    a) To establish the retesting period of Drug Substance or Drug Product.
    b) To establish the shelf life of Drug Substance or Drug Product.
    c) To establish the storage condition of Drug Substance or Drug Product.
- Because physical, chemical & microbial changes can affect effiency and security of final product.

It also plays important role in the drug development process. It explains several factors that affect the expiry dating of drug product including chemical and physical stability during preclinical, formulation stages, process development, packaging development and post marketing life.

Stability affects the quality, safety and efficacy. Change in drug stability could risk patient safety. Instability leads to chemical degradation, loss of potency, possible formation of new chemical species with potential toxic side effects.

To aid prediction of drug stability, forced or accelerated degradation is performed to elucidate potential degradation products, determine their safety and to develop analytical procedure to quantitative these new chemical species.

It may be prediction to degradative pathways of a drug under normal condition. These studies will expose the drug to potentially degrading conditions including moisture, oxygen, pH, temperature and light.

Types of stability indicating assay method (SIAM):

a) Specific Stability Indicating Assay Method:

It can be defined as “a method that is able to measure unequivocally the drug(s) in the presence of all degradation products, in the presence of excipients and additives, expected to be present in the formulation.”

b) Selective Stability Indicating Assay Method:

Whereas it can be defined as "a method that is able to measure unequivocally the drug(s) and all degradation products in the presence of excipients and additives, expected to be present in the formulation."

With the advent of International Conference on Harmonisation (ICH) guidelines, the requirement of establishment of stability-indicating assay method (SIAM) has become more clearly mandated. The guidelines explicitly require conduct of forced decomposition studies under a variety of conditions, like pH, light, oxidation, dry heat, etc. and separation of drug from degradation products. The method is expected to allow analysis of individual degradation products.

- **Various Regulatory guidelines available to carry out SIAM:**

Although FDA guidelines and ICH guidelines provide useful definitions and general information about forced degradation studies, their direction concerning the scope, timing and best practice is very general and lacking in details.

Table 1: ICH guidelines [3]

Guidelines reference	Title
Q1A(R2)	Stability testing of new DS and DP
Q1B	Photo stability of new DS & DP
Q1E	Evaluation of stability data
Q2 (R1)	Validation of analytical procedures
Q3A(R2)	Impurities in new DS
Q3B (R2)	Impurities in new DP
Q5C	Stability testing of biotech /biological products
Q6A	Specification : New DP/DS(to determine content of DS/DP)
Q7	GMP for API

Table 2: Selection of FDA and EMEA guidelines and pharmacopeia chapter referencing SIAM or forced degradation [3]

Guidelines reference	Title
USP <1086>	Impurities in official articles
USP <11>	USP reference standard

USP <1150>	Pharmaceutical stability
USP <1191>	Stability consideration in dispensing practice
USP <797>	Pharmaceutical compounding of sterile products, storage and beyond use dating
EMEA March 2001	Note for guidance on in use stability testing of human medicinal products
EMEA Dec 2004	Guidance on chemistry of new API
FDA guidance for industry	Analytical procedures and method validation

- **Techniques employed in SIAM:**

Titrimetric, spectrophotometric and chromatographic techniques have been commonly employed in analysis of stability samples.

a) Titrimetric and Spectrophotometric method :

Objective: Analysis of the drug of interest alone in the matrix of excipients, additives, degradation products, impurities, etc., and also other drugs in case of the combination products.

Advantage: Low cost and simple.

Disadvantage: Non-sensitive and non selective.

However, a few reports involving derivative spectroscopy have been published lately. [4, 5, 6]

b) Chromatographic techniques:

Advantage: Greater accuracy and sensitivity for even small quantities of degradation products produced. [7, 8, 9, 10]

Method used: TLC, HPTLC, HPLC, GC, CE.

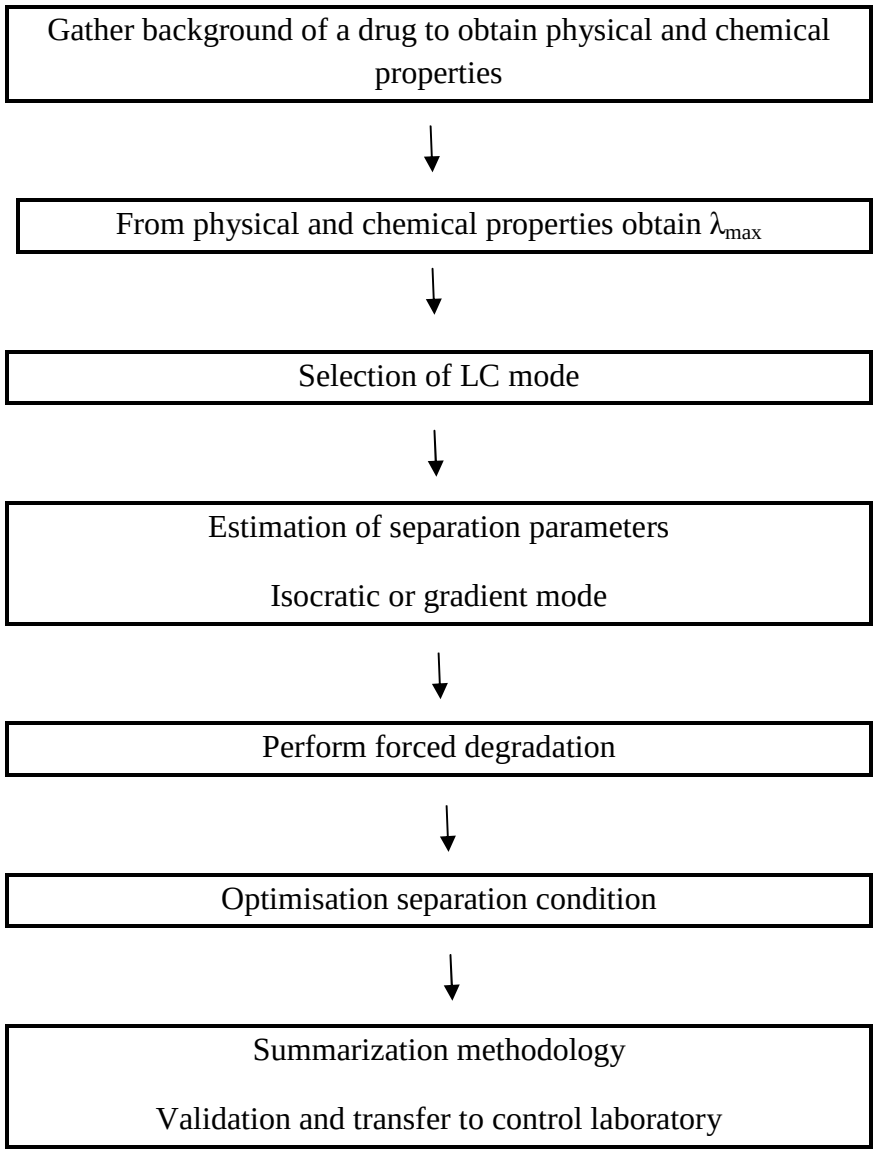
c) Miscellaneous:

A few studies have also reported the use of proton nuclear magnetic resonance (NMR) spectroscopy for the development of SIAMs. **[11, 12, 13]**

CE is the latest entry to the techniques for the development of SIAMs. It has the advantage of high sensitivity, resolution and high efficiencies with minimal peak dispersion. [14, 15, 16]

There are several publications involving use of hyphenated GC-MS [17], LC-MS [17-20], LCMS- MS [19, 20, 21], LC-NMR [19, 22] and CE-MS [23, 24] techniques for identity confirmation of known and unknown degradation products and their selective determination.

Overview of SIAM



- **Steps of Development of validated SIAMs:**

Step I: ***Critical Study of the Drug Structure to Assess the Likely Decomposition Route(S)***

There are definite functional group categories, like amides, esters, lactams, lactones, etc. that undergo hydrolysis [25] others like thiols, thioethers, etc. undergo oxidation[26] and compounds like olefins, aryl halo derivatives, aryl acetic acids, and those with aromatic nitro groups, N-oxides undergo photodecomposition. [27]

Step II: ***Collection of Information on Physicochemical Properties***

Before method development is taken up, it is generally important to know various physicochemical parameters like pKa, log P, solubility, absorptivity and wavelength maximum of the drug in question. [28]

Step III: ***Stress (Forced Decomposition) Studies***

The next step in the development of SIAM is the conduct of forced decomposition studies to generate degradation products of the drug. The ICH guideline Q1A suggests the following conditions to be employed: (i) 10 °C increments above the accelerated temperatures (e.g. 50 °C, 60 °C, etc.), (ii) humidity where appropriate (e.g. 75% or greater), (iii) hydrolysis across a wide range of pH values, (iv) oxidation and (v) photolysis. However, the guideline provides no details on how hydrolytic, photolytic and oxidative studies have to be actually performed. A comprehensive document providing guidance on the practical conduct and issues related to stress testing under variety of ICH prescribed condition has been published lately .[29,30]

Step IV*: Preliminary Separation Studies on Stressed Samples*

The stress samples so obtained are subjected to preliminary analyses to study the number and types of degradation products formed under various conditions.
Column preferred: Reversed-phase octadecyl silane column preferred first.
Mobile phase: Water– methanol or water–acetonitrile preferred first. If it doesn't satisfy SSP, then Buffer is preferred.
The **injection volume** and the **flow rate** can be suitably adjusted based on the length of the column.

Step V: ***Final Method Development & Optimization***

Subsequent to preliminary chromatographic studies, the RT and relative retention times (RRT) of all products formed should be tabulated for each reaction condition. Special attention is then paid to those components whose RT or RRT is very close. PDA spectra or LC-MS profile of such components are obtained and critically evaluated to ascertain whether the products are same or different. In the final step, a mixture of the reaction solutions is prepared, and subjected again to resolution behaviour study. Rather, only those solutions are mixed where different products are formed in sufficient quantity. Resolution in the mixture is studied closely, to see whether the resolution is similar to that obtained in individual samples. This is important to rule out any changes that can happen when reaction solutions of different pH and media (3–30% hydrogen peroxide solution) are mixed. [30]

Step VI: ***Identification & Characterization of Degradation Products, & Preparation of Standards***

Method used: MS, NMR IR, hyphenated techniques coupled with MS and Tandem MS (LC-MS-MS), LC –NMR and, Preparative TLC or Preparative HPLC.

Step VII: ***Validation of SIAMS***

Validation of analytical methods, in general, has been extensively covered in the ICH guidelines Q2A and Q2B [31, 32] in the FDA guidance [33] and by USP [34]. The main focus of validation at this stage is on establishment of specificity/selectivity, followed by other parameters like accuracy, precision, linearity, range, robustness, etc. The limits of detection and quantitation are also determined for degradation products to help in establishment of the mass balance.

Peak Purity:

It is the analysis of the main peak to assess for the presence of impurities under the main peak, is an essential part of the validation of the SIM. The determination of peak purity is more difficult than it seems as one can never be certain that a peak is truly pure. Direct evaluation can be performed in line by employing PDA detectors, LC-MS, LC-NMR, absorbance ratio method, dual wavelength ratio chromatography, second order derivative spectroscopy, spectral suppression, spectral overlay, etc. [35] PDA only works well for degradants those have different UV spectrum from that of the drug.LC MS evaluation will not work if the degradants have the same mass as is the case for the diastereomers.

1.2. Role of Phosphodiesterase Enzymes (PDEs) & their Inhibitors[36,37]

PDE enzymes break a phosphodiester bond. PDEs comprise of group of enzymes that degrade the phosphodiester bond in the 2nd messenger molecule like cyclic adenosine monophosphate (cAMP) or cyclic guanosine monophosphate (cGMP). 14 different subtypes of PDEs have been identified. They regulate the localization, duration and amplitude of cyclic nucleotide signalling within sub cellular domain. PDEs are therefore important regulators of signal transduction mediated by these 2nd messengers. Inhibitors of PDEs can prolong or enhance the effects of physiological processes mediated by cAMP or cGMP by inhibition of their degradation by PDE.

A **phosphodiesterase inhibitor** is a drug that blocks one or more subtypes of the enzyme phosphodiesterase (PDE), therefore preventing the inactivation of the intracellular second messengers cAMP and cGMP by the respective PDE subtype(s).

1.2.1 Classification of PDEs Inhibitors:

a) Non-selective phosphodiesterase inhibitors

- **Methylated xanthines and derivatives:**

- Caffeine, a minor stimulant.
- IBMX (3-isobutyl-1-methylxanthine), used as investigative tool in pharmacological research.
- Paraxanthine.
- Aminophylline.
- Pentoxifylline, a drug that has the potential to enhance circulation and may have applicability in treatment of diabetes, fibrotic disorders, peripheral nerve damage, and microvascular injuries.
- Theobromine.
- Theophylline, a bronchodilator.

- **Methylated xanthines act as both:**

- Competitive nonselective phosphodiesterase inhibitors which raise intracellular cAMP, activate PKA, inhibit TNF-alpha and leukotriene synthesis, and reduce inflammation and innate immunity.
- Nonselective adenosine receptor antagonists.

But different analogues show varying potency at the numerous subtypes, and a wide range of synthetic xanthine derivatives (some nonmethylated) have been developed in the search for compounds with greater selectivity for phosphodiesterase enzyme or adenosine receptor subtypes.

b) Selective phosphodiesterase inhibitors

➢ ***PDE1 selective inhibitors***

- Vinpocetine.

➢ ***PDE2 selective inhibitors***

- EHNA (**e**rythro-9-(2-**h**ydroxy-3-**n**onyl) **adenine**).
- Anagrelide.

➢ ***PDE3 selective inhibitors***

- Enoximone and milrinone, used clinically for short-term treatment of cardiac failure. These drugs mimic sympathetic stimulation and increase cardiac output.

PDE3 is sometimes referred to as cGMP-inhibited phosphodiesterase.

➢ ***PDE4 selective inhibitors***

- Drotaverine.
- Mesembrine, an alkaloid from the herb Sceletium tortuosum.
- Rolipram, used as investigative tool in pharmacological research.
- Ibudilast, a neuroprotective and bronchodilator drug used mainly in the treatment of asthma and stroke. It inhibits PDE4 to the greatest extent, but also shows significant inhibition of other PDE subtypes, and so acts as a selective PDE4 inhibitor or a non-selective phosphodiesterase inhibitor, depending on the dose.
- Piclamilast, a more potent inhibitor than rolipram.
- Luteolin, supplement extracted from peanuts that also possesses IGF-1 properties.

PDE4 is the major cAMP-metabolizing enzyme found in inflammatory and immune cells. PDE4 inhibitors have proven potential as anti-inflammatory drugs, especially in inflammatory pulmonary diseases such as asthma, COPD, and rhinitis. They suppress the release of cytokines and other inflammatory signals, and inhibit the production of reactive oxygen species. PDE4 inhibitors may have antidepressive effects and have also recently been proposed for use as antipsychotics.

➢ ***PDE5 selective inhibitors***

- Sildenafil, tadalafil, vardenafil, and the newer udenafil and avanafil selectively inhibit PDE5, which is cGMP-specific and responsible for the degradation of cGMP in the corpus cavernosum. These phosphodiesterase inhibitors are used primarily as remedies for erectile dysfunction, as well as having some other medical applications such as treatment of pulmonary hypertension.
- Dipyridamole also inhibits PDE5. This results in added benefit when given together with NO or statins.

1.3 INTRODUCTION TO DRUG PROFILE [38, 39, 40]

Drotaverine (also known as **drotaverin**) is an antispasmodic drug, structurally related to papaverine. Drotaverine is a selective inhibitor of phosphodiesterase 4 and has no anticholinergic effects. A small 2003 study found drotaverine to be nearly 80% effective in treating renal colic. It has also been used to accelerate labor.

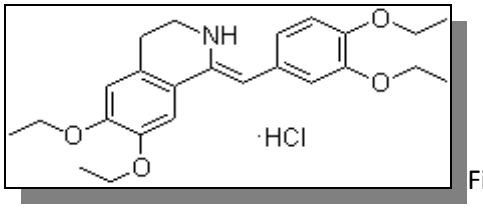


Figure 1: Drotaverine Hydrochloride

- Molecular Formula : $C_{24}H_{31}NO_4 \cdot HCl; C_{24}H_{32}ClNO_4$
- Molecular Weight : **433.97**
- CAS Registry Number : **985-12-6**
- Melting point : **208-212 ºC**

➢ **IUPAC Name :**

- 6,7,3,4'-Tetraethoxy-1-benzal-1,2,3,4-tetrahydroisoquinoline hydrochloride .
- (Z)-1-(3,4-diethoxybenzylidene)-6,7-diethoxy-1,2,3,4-tetrahydroisoquinoline.
- 1-(3,4-Diethoxybenzylidene)-6,7-diethoxy-1,2,3,4-tetrahydroisoquinoline hydrochloride.

- (1Z)-1-((3,4-diethoxyphenyl)methylidene)-6,7-diethoxy-3,4-dihydro-2H-isoquinoline hydrochloride.

➢ **Other Names:**

- Isodihydroperparine hydrochloride.
- Drotaverin HCl, Drotaverinium chloride.

➢ **Appearance:** Yellowish and greenish shade crystalline powder.

➢ **Solubility :** Moderately soluble in Water, soluble in 96% Ethanol, easily soluble in Chloroform.

➢ **pH of 1% aqueous solution :** 3.5-5.5

➢ **Stability & Reactivity**

- **Stability:** Stable under normal conditions. May discolour on exposure to light.
- **Incompatible materials:** Strong oxidizing agents.
- **Decomposition products:** Carbon monoxide, Carbon dioxide, Nitrogen oxides, Hydrogen chloride.
- **Polymerization:** Has not been reported.
- **NFPA Ratings :** Health:2 , Flammability:0 , Reactivity:1

➢ **Safety**

- **Hazard notes:** Harmful if swallowed. Target organ(s): Smooth muscle.
- **Eye:** May cause eye irritation.
- **Skin:** May be harmful if absorbed through skin. May cause skin irritation.
- **Inhalation:** Harmful if inhaled. Material may be irritating to mucous membranes and respiratory tract.

➢ **Pharmacokinetic data :**

- **Peak concentrations**: 1 to 3 hours after oral dose.
- **Oral bioavailability**: 25 to 91% and its metabolites are 80% to 95% protein bound volume of distribution 193 to 195 litres.

- **Metabolism** : Hepatic.
- **Half life** : 7 to 12 hours.
- **Excretion** : Fecal and renal.
- **Routes of administration**: Oral, intravenous.

The pharmacokinetics and bioavailability of drotaverine were studied in 10 healthy volunteers. The pharmacokinetic parameters, such as elimination half-life, plasma clearance, renal clearance and apparent volume of distribution, were not influenced by the route of drug administration. The drug was mainly eliminated by non-renal routes since renal clearance accounted for only 0.31 +/- 0.13% of the total plasma clearance. The absolute bioavailability was variable and ranged from 24.5-91% with a mean of 58.2 +/- 18.2% (mean +/- SD). It is suggested that the high variation in the bioavailability of drotaverine HCl after oral administration may result in significant interindividual differences in therapeutic response. (41)

Bioavailability of drotaverine from capsule and tablet preparations in healthy volunteers studied by Dyderski S, Grześkowiak E, Drobnik L, Szałek E, Balcerkiewicz M, Dubai V.(42)

- **Pharmacology:**

Category: ***A Spasmolytic Agent***

Drotaverine hydrochloride is an analogue of papaverine with the smooth muscle relaxant properties. It is non-anticholinergic & antispasmodic, which selectively inhibits phosphodiesterase IV and is accompanied by a mild calcium channel-blocking effect.

1) Drotaverine hydrochloride can be used in **Acute Colicky Pain** caused by renal and ureteric stones.(43)
2) Drotaverine hydrochloride can be used for **Augmentation of Labor**.(44)
   Drotaverine hydrochloride is used for acceleration of labor and relief of labor pains. There are no adverse fetal effects, but atonic postpartum haemorrhage is more common.
3) Efficacy of drotaverine is seen in **Irritable Bowel Syndrome**.(45)
4) **Relaxant Effect** of Drotaverine is seen in **human Isolated Ureteral Rings.(46)**

- **Application :** Spasm smooth muscles of internal organs (cardio and pilorospazm), chronic gastroduodenit, gastric ulcer disease and gastrointestinal cancer, renal colic, chronic cholecystitis, psoriasis spastic bowel, intestinal colic because of the delay after gas operations, colitis, flatulence.

- **Contraindications :**
  - In glaucoma.
  - Restrictions apply to: Enlarged prostate gland expressed in atherosclerotic coronary arteries.
  - Drotaverine interacts with the L-type Ca^{2+} channel in pregnant rat uterine membranes
  - Severe renal/hepatic/cardiac dysfunction, Porphyria.
  - May attenuate the action of levodopa.
- Concurrent use of analgesics, antimuscarinics or benzodiazepines is **beneficial**. Additive beneficial effect with concurrent use of analgesics, antimuscarinics or benzodiazepines.

- **Cautions:** Drotaverine is generally well tolerated. Reported adverse effects include porphyria, hypotension, tachycardia, headache, vertigo, and nausea.

- **Adverse reactions:**

a) ***Cardiovascular:***

- Hypotension has been reported following use of drotaverine (Sharma et al, 2001).
- Tachycardia (4%) has occurred in nulliparous women in active labor receiving drotaverine, although 4% of those receiving no medication also exhibited tachycardia (Sharma et al, 2001).

b) ***Central nervous system:***

- Headache, Vertigo, Insomnia & Dizziness have been reported in patients receiving Drotaverine (Sharma et al, 2001).

c) ***Endocrine/Metabolic:***

- Metabolic effects: Acute attacks of Porphyria have been associated with drotaverine use (Sweetman, 2002).

d) ***Gastrointestinal :***

- Nausea has rarely been reported following use of drotaverine (Sharma et al, 2001).
- Constipation.
- Vomiting.

e) ***Kidney/Genitourinary :***

- Cervical tearing has occurred following use of drotaverine (Sharma et al, 2001).

f) ***Teratogenicity/effects in pregnancy teratogenicity :***

- Effects in human pregnancy are unknown. Datas are lacking.

- **Dosage:**
  - Adults: 40-80 mg thrice 3 daily.
  - Children**:** 1-5 years 20mg, 3-4 times daily. 6-12 year : 40mg, thrice daily
- **Overdosing:** AV blockade, cardiac arrest, respiratory paralysis centre.

- **Marketed preparation :**

DITO (Lifecom Pharma), DIVER (Corvin), DOTARIN (Elder H. Care), DROTARIX (Cadex Lab) etc. It is available in combination with NSAID like Mafenamic acid, PCM; antacids like Omeprazole etc.

1.4 INTRODUCTION TO HPLC[47]

It is commonly known as:

- ***High Performance Liquid Chromatography (HPLC)***
- ***High Price Liquid Chromatography (HPLC)***
- ***High Speed Liquid Chromatography (HSLC)***
- ***High Efficiency Liquid Chromatography (HELC)***

LC is an analytical technique that is useful for separating ions or molecules that are dissolved in a solvent. If the sample solution is in contact with a second solid or liquid phase, the different solute will interact with the other phase to differing degree due to difference in absorption, ion exchange, partitioning or size. These differences allow the mixed component to be separated from each other by using these differences to determine the transit time of solutes through column.

- **Method in chromatography**

1) Adsorption chromatography

- Normal phase chromatography
- Reversed phase chromatography
- Ion exchange chromatography
- Affinity chromatography
- Hydrophobic interaction chromatography (HIC)

2) Partition chromatography

- Gas chromatography
- Liquid liquid partition chromatography

3) Size exclusion chromatography

- Gel permeation chromatography
- Gel chromatography
- Gel filtration chromatography

- **Reversed Phase Chromatography**

a) Mechanism:

Retention by interaction of non polar hydrocarbon chain of stationary phases with non polar parts of the sample molecules.

HPLC instrument consists of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Compounds are separated by injecting a plug of the sample mixture onto the column.

b) Schematic diagram:

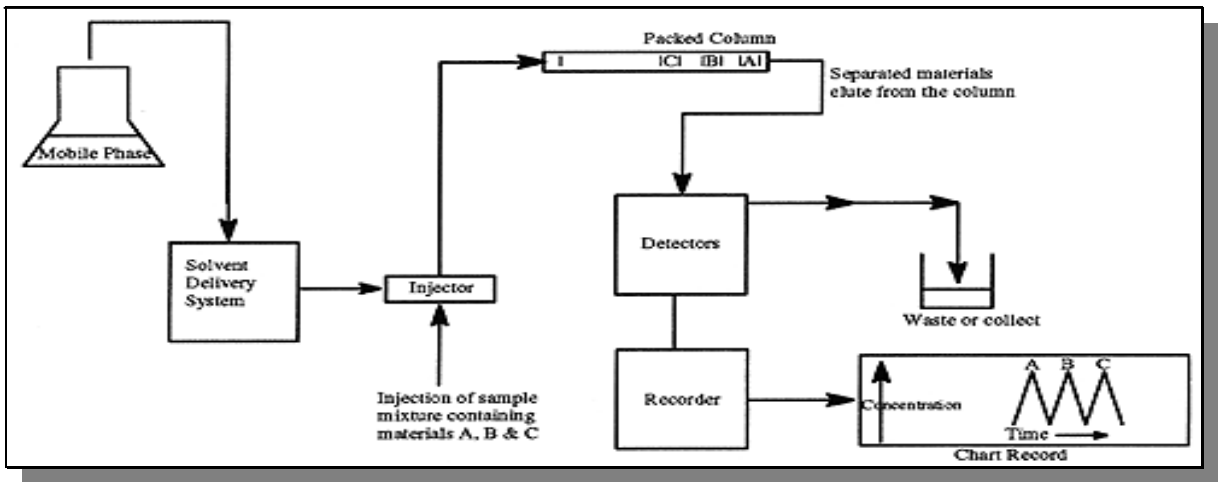


Figure 2: Schematic diagram of HPLC

c) Mobile phase :

- Methanol (CH_3OH)
- Acetonitrile (CH_3CN)
- Water (H_2O)
- Buffer
- Tetrahydrofuran (THF)

d) Solvent degassing:

Why solvent degassing is so important?

- To get the stable baseline.
- To enhance sensitivity of detector.
- Reproducibility of injection for quantification.
- For stable pump operation.

Methods for degassing:

- Sparging (online degassing).
- Heat (offline degassing).

- Vaccum (offline degassing).
- Sonication (offline degassing).

e) Solvent delivery systems / Pumps:

Types of the pumps:

1) Syringe pumps (screw driven)
2) Reciprocating pump
    - Single piston reciprocating pump
    - Dual piston reciprocating pump
    - Reciprocating diaphragm pump

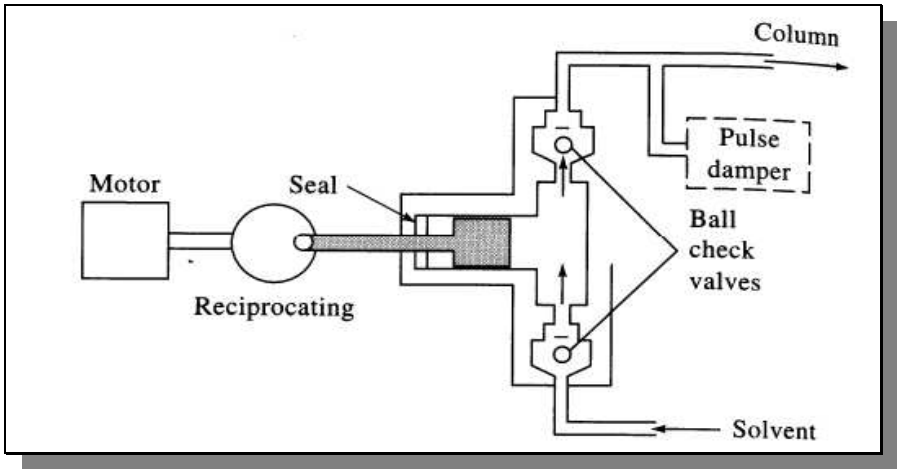


Figure 3: Single piston reciprocating pump

3) Pneumatic pump:
    - Direct pressure pump
    - Amplifier pump

Table 3: Merits & Demerits of HPLC pumps

Types of pump	Advantage	Disadvantage
Syringe (Position displacement)	1) Pulse free delivery at high pressure. 2) FR independent of viscosity of MP.	Limited solvent capacity. Change in solvent is inconvenient.

Reciprocating pump	1) FR independent of viscosity of MP. 2) Suitable for continuous operation.	Detection of noise due to pulsating out flow.
Pneumatic pump	1) Rugged 2) Inexpensive 3) easy to operate 4) Pulse free	FR dependent of viscosity of MP. Change in solvent is inconvenient. Gradient operation is difficult.

Desired Pump characteristics:

- Pressure in PSI : 3000-5000
- FR in ml/min : 0.5-2 (analytical), 0-10 (preparative)
- Accuracy : ±5%
- Reproducibility : ±1 %
- Solvent storage : 200-500 ml
- Pulse free delivery of mobile phase : Essential

f) HPLC columns:

- It is the heart of the HPLC.
- Stable and high performance column is essential requisite for rugged, reproducible method.

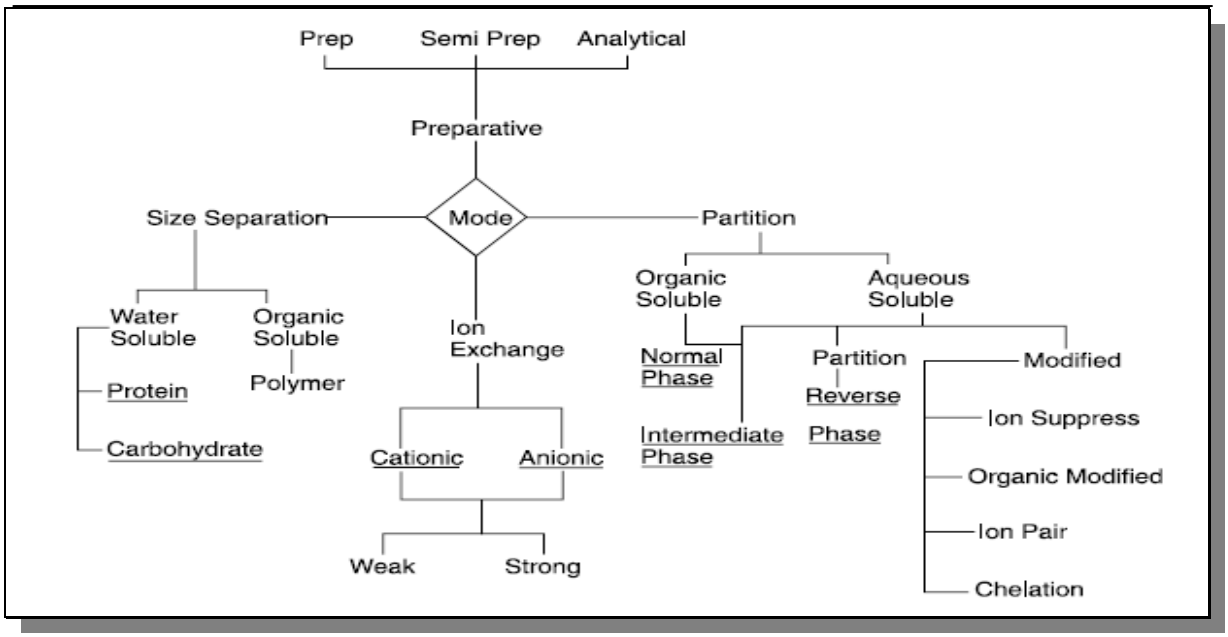


Figure 4: Guide to Column selection

- Separation Column: Usually stainless steel tubing with i.d. of 2-6 mm (analytical separations).
- Larger columns (e.g. 30-200 mm i.d.): Preparative separations in the range of 100 mg to 1 Kg can be performed.

A fine grained chromatographic material (e.g. silica gel or RP-18), serves as stationary phase with particle size of 5-10 μm (analytical separations) and 10-50 μm (preparative separations).

To overcome the flow resistance of stationary phase, mobile phase must place under a relatively high pressure up to 100 bars.

Factor influencing the life of HPLC column:

- Loss of bonded phase.
- Dissolution of column surface.
- Pressure increase.
- Column channelling.

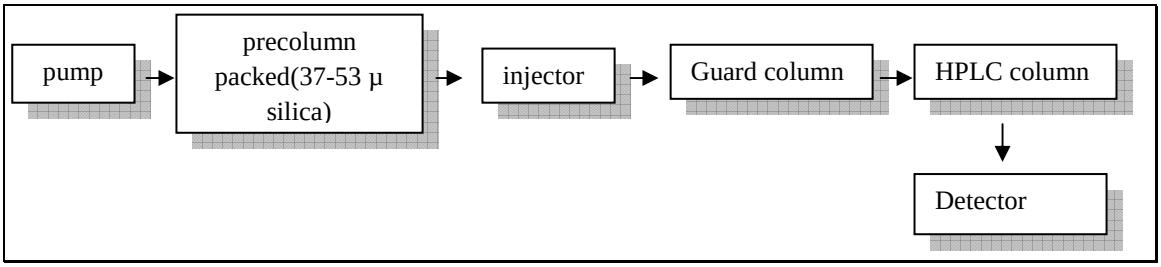
g) Stationary phase :

Table 4: Stationary phase in LC

Group	Type	Particle diameter (µ)
Amino	Normal	5 & 10
Nitrile	Normal	5 & 10
Amine & Nitrile	Normal	40
Octyl	Reverse phase	3,4,5 & 10
Octadecyl	Reverse phase	4,5,10 & 40

h) Precolumn :

Some of operating conditions which cause acute dissolution of silica are such as pH>3 or < 2, temperature >50°C, concentrated buffer and ion pair reagents. This dissolution leads to column bed subsidence resulting in formation of void at top of the column which can lead to broadening of peak and increase pressure. Precolumn fitted between pump and injection valve ensures that the mobile phase is fully saturated with silicates ions prior to the sample injection. Thus the use of precolumn shall reduce adverse effects of low or high ph mobile phase. It extends life of column.



i) Sample Injection System:

Injection systems, includes manual injector, standard auto sampler, high-performance auto sampler, high-performance auto sampler SL plus, micro well-plate auto sampler, preparative auto sampler and dual-loop auto sampler as well as the thermostat.

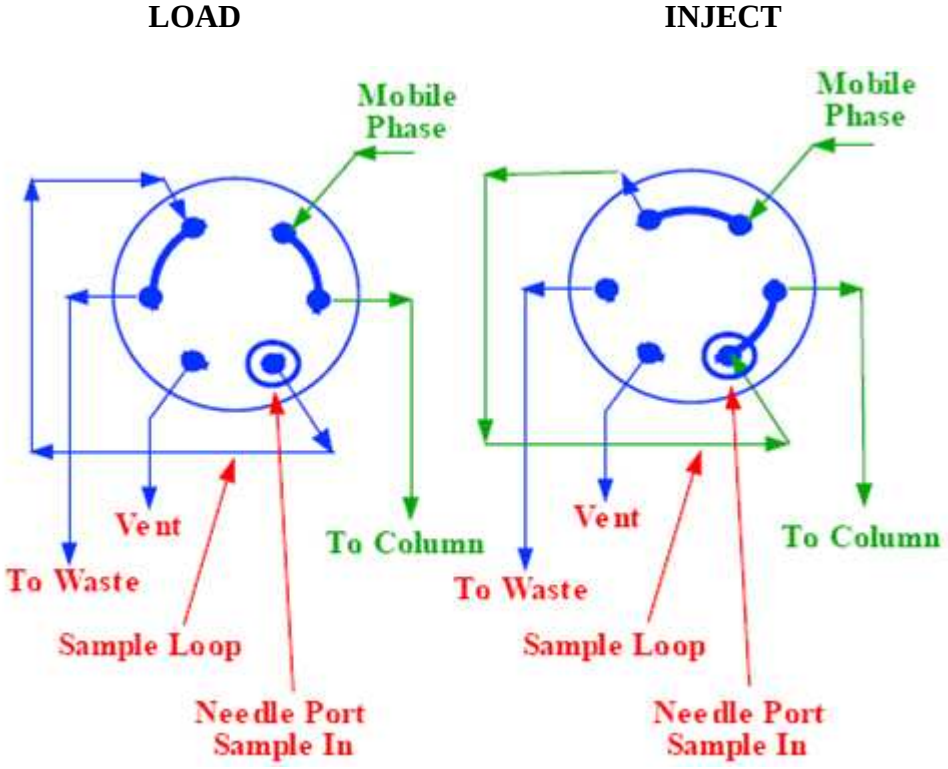


Figure 5: Sample Injection system in HPLC

j) Guard column :

The purpose of the guard column is to protect expensive of analytical column by removing partical garbage and strongly irreversible retained sample component which decrease the life time of analytical column.

k) Detectors :

It is the eye of the LC. It measures the separated components.

Two types of detectors:

1) **Bulk property detectors**: They are based on some bulk properties of eluent, such as RI and are not suitable for gradient elution and are usually less sensitive than solute property detectors.

2) **Solute property detectors:** Performed by measuring some types of physical or chemical property that is specific to solute only. So can be used with gradient elution.

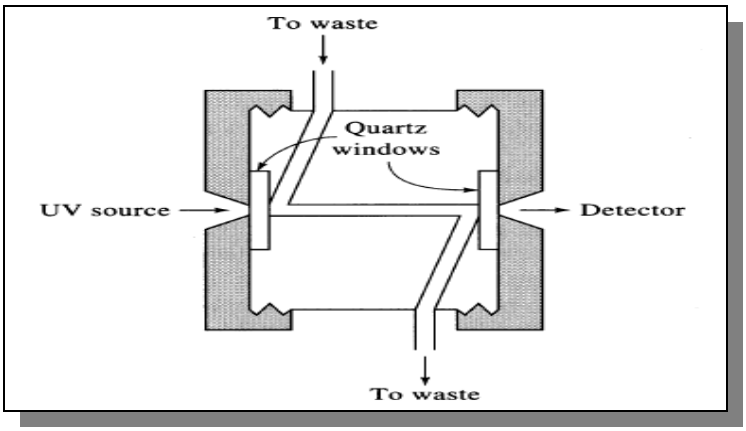


Figure 6: schematic diagram of UV VIS detector

- UV VIS Detector
- Variable wave length detector
- Photo diode array (PDA)
- Refractive index (RI)
- Evaporating light scattering detector(ELSD)
- Chiral detector
- Conductivity detector
- Polarimeter for enantiomer
- Viscometer for polymers
- Radioisotope for flow detector

Table 5: Detectors and their applications

Detector	Analyte	Solvent requirement	Comment
UV VIS	Analyte with chromophore.	UV grade non UV absorbing.	It has a degree of selectivity and useful for many HPLC application.
Fluorescence	Fluorescent compound.	UV grade non UV absorbing.	High selectivity and sensitivity. Often used to

			analyze derivatized compound.
RI	Compound with different RI to that of MP.	Cannot run MP gradient.	Virtually a universal detector but has limited sensitivity.
Conductivity detector	Charged or polar compound.	MP must be conducting.	Excellent for ion exchange methods.
Electrochemical detector	Readily oxidized or reduced compound especially biological samples.	MP must be conducting.	Very selective and sensitive.
ELSD	Virtually for all compound.	Must use volatile solvent and volatile buffer.	A universal detector which is highly sensitive. Not selective.
MS	Broad range of compound.	Must use volatile solvent and buffer.	Highly sensitive and powerful 2D analytical tool.

The parameters that are affected by the changes in chromatographic conditions are,

- Resolution (R_s),
- Capacity factor (k'),
- Selectivity (α),
- Column efficiency (N) and
- Peak asymmetry factor (As).

Table 6: Separation Goals in HPLC Method Development

Goal	Comment
Resolution (Rs)	Rs should be greater than 1.5
Separation time	<5-10 min for routine analysis
Quantitation	≤ 2% for assays, ≤ 5% for less demanding analysis
Pressure	< 150 bar desirable, <200 bar is usually essential

Peak height	Narrow peaks are desirable for large S/N
Solvent consumption	Minimum mobile phase use per run is desirable

1.5 QUANTIFICATION IN LC[48]

Peak height or peak area measurements only provide a response in terms of detector signal. This response must be related to the concentration or mass of the compound of interest. To accomplish this, some type of calibration must be performed.

The four primary techniques for Quantification are:

- Normalized peak area method
- External Standard method
- Internal Standard method
- Method of Standard addition

➢ **Normalized peak area method**

The area percent of any individual peak is referred to the normalized peak area. This technique is widely used to estimate the relative amounts of small impurities or degradation compounds in a purified material and in this method; the response factor for each component is identified.

➢ **External Standard method**

This method includes injection of both standard and unknown and the unknown is determined graphically from a calibration plot or numerically using response factors.

A response factor (R_f) can be determined for each standard as follows:

$$R_f = \frac{\text{Standard Area (Peak height)}}{\text{Standard Concentration}}$$

The external standard approach is preferred for most samples in HPLC that do not require extensive sample preparation.

➢ **Internal Standard method**

The internal standard is a different compound from the analyte but one that is well resolved in the separation. The internal standard should be chosen to mimic the behaviour of the sample compound. One of the main reasons for using an internal standard is for samples requiring significant pre-treatment or preparation.

Response Factor is used to determine the concentration of a sample component in the original sample. The response factor is the ratio of peak areas of sample component (Ax) and the internal standard (A_{ISTD}). It can be calculated using the formula,

$$R_f = \frac{Ax}{A_{ISTD}}$$

On the basis of the response factor and strength of the internal standard (N_{ISTD}), the amount of the analyte in the original sample can be calculated using the formula,

$$X = \frac{A_S}{R_F.A_{1STD}} x N_{1STD}$$

- **Method of Standard addition:**

The method of standard addition can be used to provide a calibration plot for quantitative analysis. It is most often used in trace analysis. An important aspect of the method of standard addition is that the response prior to spiking additional analytes should be high enough to provide a reasonable S/N ratio (>10), otherwise the result will have poor precision.

1.6. GETTING STARTED ON METHOD DEVELOPMENT[47,49]

"Best column, best mobile phase, best detection wavelength, efforts in separation can make a world of difference while developing LC method for routine analysis. Determining the ideal combination of these factors assures faster delivery of desired results – a validated method of separation.**"**

- **The Best Mobile Phase**

In reverse-phase chromatography, the mobile phase is more polar than the stationary phase. Mobile phase in these systems is usually mixtures of two or more individual solvents with or without additives or organic solvent modifiers. Separations in these systems are considered to be due to different degrees of hydrophobicity of the solutes. The polarity of organic modifier and its proportion control the rate of elution of the components in the mobile phase. The rate of elution is increased by increasing the polarity. The simple alteration of composition of the mobile phase or of the flow rate allows the rate of the elution of the solutes to be adjusted to an optimum value and permits the separation of a wide range of the chemical types. First isocratic run followed by gradient run is preferred.

Since the mobile phase governs solute-stationary phase interaction, its choice is critical.

- Practical considerations dictate that it should not degrade the equipment or the column packing. For this reason, strong acids, bases and halide solutions should be avoided.
- Chemical purity of solvents is an important factor. Since large volumes of solvent are pumped through the column, trace impurities can easily concentrate in column and eventually be detrimental to the results. Spectro or HPLC grade solvents are recommended.
- Volatility should be considered if sample recovery is required.
- Viscosity should be less than 0.5 centipoises, otherwise higher pump pressures are required and mass transfer between solvent and stationary phase will be reduced.
- LC/MS-only volatile buffers.

➢ **The Best Detector**

UV-visible detectors are the most popular as they can detect a broad range of compounds and have a fair degree of selectivity for some analytes. As shown in detector table.

➢ **The Best Column Length**

Method development can be streamlined by starting with shorter columns; 50, 100 or even 150mm long. This is simply because they have proportionally shorter run times as illustrated in Figure.

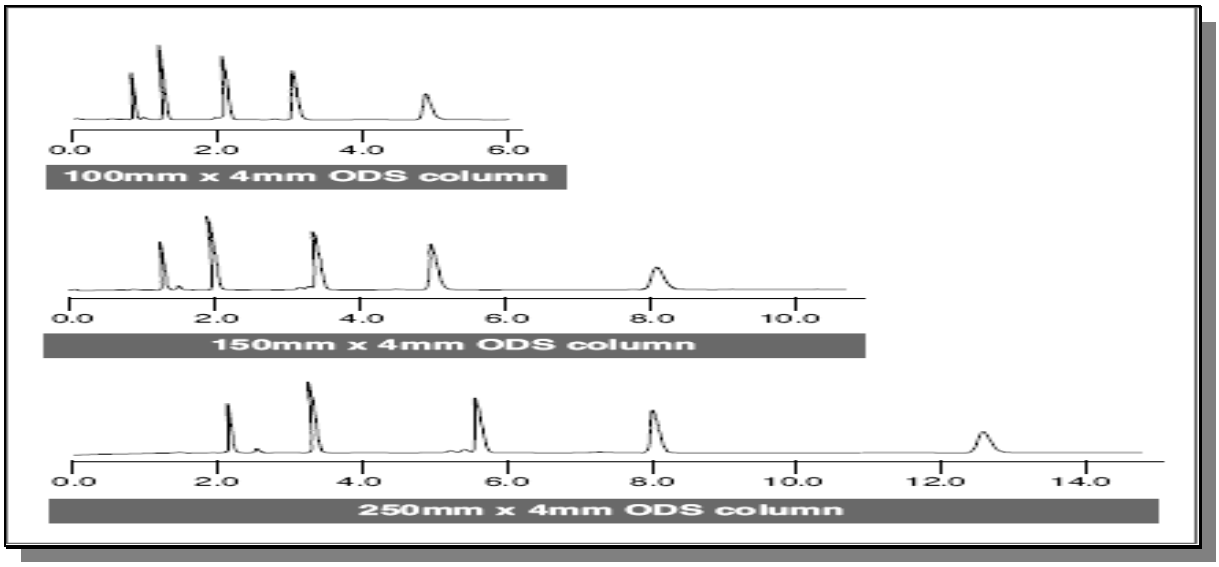


Figure 7: Effect of Column length

- **The Best Stationary Phase**

C_8 phase (reversed phase) can provide a further time saving over a C_{18}, as it does not retain analytes as strongly as the C_{18} phase.

- **Retention**

Analytes may be too strongly retained (producing long run times). If this occurs, the solvent strength should be increased. In reverse phase analysis this means a higher % of organic solvent in the mobile phase.

- **Poor Separation**

Analytes often co-elute with each other or impurities. To overcome this, the analysis should be run at both higher and lower solvent strengths so the best separation conditions may be determined. Varying solvents may help - try methanol instead of acetonitrile for reversed phase analysis. Using buffers and modifying the pH (within the column's recommended pH range) can also assist the separation.

- **Peak Shape**

This is often a problem, especially for basic compounds analyzed by reversed phase HPLC. To minimize any potential problems always use a high purity silica phase such as Wakosil II. These modern phases are very highly deactivated so secondary interactions with the support are minimal. Buffers can be used effectively to give sharp peaks. If peak shape remains a problem, use an organic modifier such as triethylamine, although this should not be necessary with modern phases like Wakosil. One point often forgotten is the effect of temperature changes on a separation. To maximize the reproducibility of a method, it is best to use a column heater to control the temperature of the separation. A temperature of 35 – 40°C is recommended.

- **Buffer selection**

In reverse phase HPLC, the retention of analytes is related to their hydrophobicity. The more hydrophobic the analyte, the longer it is retained. When an analyte is ionized, it becomes less hydrophobic and, therefore, it retention decreases. When separating mixtures containing acid and/or bases by reversed phase HPLC, it is necessary to control the pH of mobile phase using appropriate buffer in order to achieve reproducible results.

When separating acids and bases a buffered mobile phase is recommended to maintain consistent retention and selectivity. A buffered mobile phase, by definition, resists changes in pH so that the analytes and silica will be consistently ionized, resulting in reproducible chromatography. If the sample is neutral, buffers or additives are generally not required in the mobile phase. Acids or bases usually require the addition of a buffer to the mobile phase. For basic or cationic samples, "less acidic" reverse-phase columns are recommended and amine additives for the mobile phase

may be beneficial. Optimum buffering capacity occurs at a pH equal to the pKa of the buffer. Beyond that, buffering capacity will be inadequate.

Buffers play an additional role in the reproducibility of a separation. The buffer salts reduce peak tailing for basic compounds by effectively masking silanols. They also reduce potential ion-exchange interactions with unprotonated silanols (Figure). To be most effective, a buffer concentration range of 10 - 50 mM is recommended for most basic compounds.

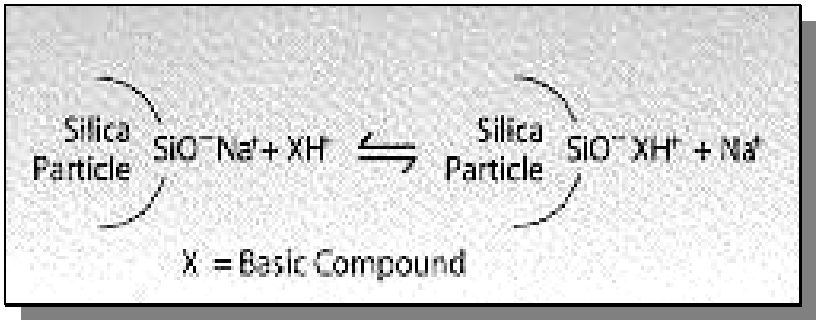


Figure 8: Peak Tailing Interaction

Table 7: Commonly used Buffers for RP- HPLC

Buffer	**P^{Ka} (25°C)**	**Maximum Buffer Range**	**UV Cut off (nm)**
TFA	0.3	-	210
Phosphate,p^{K}_{1} H_2PO_4	2.1	1.1-3.1	< 200
Phosphate,p^{K}_{2} HPO_4^{2-}	7.2	6.2-8.2	< 200
Phosphate,p^{K}_{3} PO_4^{3-}	12.3	11.3-13.3	< 200
Citrate, p^{K}_{1} $C_3H_5O(COOH)_2(COO)^{1-}$	3.1	2.1-4.1	230
Citrate, p^{K}_{2} $C_3H_5O(COOH)_1(COO)^{2-}$	4.7	3.7-5.7	230
Citrate, p^{K}_{3} $C_3H_5O(COO)^{3-}$	6.4	4.4-6.4	230

Carbonate, pK_1 HCO_3^{1-}	6.1	5.1-7.1	< 200
Carbonate, pK_2 CO_3^{2-}	10.3	9.3-11.3	> 200
Formate	3.8	2.8-4.8	210
Acetate	4.8	3.8-5.8	210
Ammonia	9.3	8.3-10.3	200
Borate	9.2	8.2-10.2	N/A
TEA	10.8	9.8-11.8	< 200

- **Selection of pH**

The pH range most often used for reversed-phase HPLC is 2 - 8 and can be divided into low pH (2 - 4) and intermediate pH (4 - 8) ranges. Each range has a number of advantages. Low pH has the advantage of creating an environment in which peak tailing is minimized and method ruggedness is, maximized. For this reason, operating at low pH is recommended.

At a mobile phase pH greater than 7, dissolution of silica can severely shorten the lifetime of columns packed with silica-based stationary phases.

The pKa value (acid dissociation [ionization] constant) for a compound is the pH at which equal concentrations of the acidic and basic forms of the molecule are present in aqueous solutions. Analytes may sometimes appear as broad or tailing peaks when the mobile phase pH is at, or near, their pKa values. A more rugged mobile phase pH will be nearby analyte pKa. This shifts the equilibrium so that 99% of the sample will be in one form. The result is consistent chromatography.

Dramatic changes in the retention and selectivity (peak spacing) of basic and acidic compounds can occur when the pH of the mobile phase is changed. This is often a result of different interactions between the column and the analytes when the ionization of these compounds changes. It is important to evaluate these changes when a method is developed in order to select the mobile phase pH that provides the most reproducible results.

1.7. ANALYTICAL METHOD VALIDATION TERMINOLOGY:

- **Definition:**

Validation is a process of establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics.

Method validation is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose. A successful Validation guarantees that both the technical and regulatory objectives of the analytical methods have been fulfilled. The transfer of a method is best accomplished by a systematic method validation process. The real goal of validation process is to challenge the method and determine limits of allowed variability for the conditions needed to run the method.

- **Objective of validation:**

The objective of validation of analytical procedure is to demonstrate that it is suitable for its intended purpose. Validation is documented evidence, which provide a high degree of assurance for specific method. Any developed method may be influenced by variables like different elapsed assay times, different days, reagents lots, instruments, equipments, environmental conditions like temperature, etc so it is expected that after the method has been developed and before it is communicated or transferred from one lab to the other, it is properly validated and the result of validity tests reported.

Two steps are required to evaluate an analytical method.

1) First determine the classification of the method.

2) The second step is to consider the characteristics of the analytical method

For analytical method validation of pharmaceuticals, guidelines from the International Conference on Harmonization (ICH), United States Food and Drug Administration (US FDA), American Association of Official Analytical Chemists (AOAC)United States Pharmacopoeia (USP), and International Union of Pure and Applied Chemists (IUPAC) provide a framework for performing such validations in a more efficient and productive manner.[50]

The primary objective of validation is to form a basis for written procedure for production and process control which are designed to assure that the drug products have the identity, strength, quality and purity they purport or are represented to possess quality, safety and efficacy must be designed to build into the product. Each

step of the manufacturing process must be controlled to maximize the probability that the finished products meet all quality and design specification.

Table 8: Comparison of analytical parameters required for the assay validation [50]

USP general chapters <1225>	ICH Q2A	FDA reviewer guidelines
Accuracy	Accuracy	Accuracy
Precision No	Precision Repeatability	Precision Repeatability
No	Intermediate precision	Intermediate precision
No	No	Reproducibility
Specificity	Specificity	Specificity/ selectivity
Detection limit	Detection limit	Detection limit
Quantification limit	Quantification limit	Quantification limit
Linearity	Linearity	Linearity
Range	Range	Range
Ruggedness	No	No
Robustness	Robustness	Robustness
System suitability	System suitability	System suitability

- **ICH guidelines**: Q2A and Q2B were developed within the expert working group of the requirement for the registration of pharmaceuticals for the human use. [50]

- ***Q2A:***

It includes the validation of analytical procedure divided in to 3 categories.

1. Identification test
2. Quantification test for impurity content
3. Quantification test for API

The objective of the analytical procedure needs to e clearly understood since this will govern the validation characteristic that needs to be evaluated.

- ***Q2B:***

It is complementary to Q2A, which presents a discussion of characteristic that should be considered during the validation of analytical procedure. It gives recommendation on how to consider the various validation characteristic of each analytical procedure.

It includes accuracy, precision, repeatability, intermediate precision, specificity, detection limit, quantification limit, linearity, range, robustness and system suitability.

Table 9: ICH validation characteristics versus type of analytical procedures [50]

Type of analytical procedure	Identification	Impurity testing		Assay
		Quantification	limit test	
Accuracy	No	Yes	No	Yes
Precision				
Repeatability	No	Yes	No	Yes
Intermediate precision	No	Yes	No	Yes
Specificity	Yes	Yes	Yes	Yes
LOD	No	Yes	Yes	No
LOQ	No	Yes	No	No
Linearity	No	Yes	No	Yes
Range	No	Yes	No	Yes

Definition of validation parameters is based in the requirement stipulated in ICH guidelines in Q2A, Q2B, FDA review guidelines and USP general characteristics <1225>.

The different parameters of analytical method development are discussed below as per ICH guideline: - [32, 33, 34, 35, 50]

1) **Specificity:**

Specificity is the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Method:

- When the impurities are available: Spiking of pure substance (drug substance or drug product) with appropriate levels of impurities/excipients and demonstrate the result is unaffected.
- When the impurities are not available: Comparing the test results of sample containing impurities or degradation product to second well-characterized procedure. These comparisons should include sample under relevant stress condition.
- In chromatographic method: Peak purity test to be done by diode array and mass spectrometry.

Expression/calculation:

- Proof of discrimination of analyte in the presence of impurities. e.g. for chromatography chromatogram should be submitted.
- Peak purity test helps in demonstrating that the peak is not attributable to more than one component.
- For assay two results should be compared and for impurity tests two profiles should be compared.

Acceptance criteria:

a) **Interference from blank, placebo and impurities:**
   - There should not be any interference from blank, placebo and impurities peak with the main peaks.
   - Peak purity factor for the main peaks in standard preparation, unspiked sample preparation and spiked sample preparation with known impurities should be equal to or more than 995.
   - Assay difference of spiked and unspiked samples should not be more than 2.0% absolute.

b) **Interference from degradation products by stress study:**
   - Degradation impurities in all degraded API preparations and sample preparations should be separated from the main peak.

- Peak purity factor for the main peaks in all unstressed and degraded API and sample preparations should be equal to or more than 995.

2) Linearity:

The linearity of an analytical procedure is its ability (within given range) to obtain test results, which are directly proportional to the concentration (amount) of analyte in the sample.

Method:

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot
- Appropriate statistical methods

Recommendation:

- Minimum of 5 concentrations are recommended

Expression/calculation:

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

Acceptance criteria:

- The correlation co-efficient (r) value should not be less than 0.999 over the working range.

3) Range:

The range of analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

Method:

Drug (different dilution) and/or separately weighed synthetic mixture. Measurement of response and plot response vs. concentration of analyte and demonstration of linearity by

- Visual inspection of plot

- Appropriate statistical methods

Recommendation:

- Assay of drug/finished product: 80 – 120% of test concentration.
- For content uniformity: 70 – 130% of test concentration.
- For dissolution testing: ± 20% over specified range.
- For impurity: from reporting level to 120% of specification.

Expression/calculation:

- Correlation coefficient, y-intercept, slope of regression line, residual sum of squares.

Acceptance criteria:

- Not specified

4) Accuracy:

The accuracy of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Method:

- Application of procedure to analyze synthetic mixture of known purity.
- Comparison of result with already established procedure.
- Accuracy may be inferred once precision, linearity and specificity have been established.

Recommendation:

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates

Expression/calculation:

- Percent recovery by the assay of known added amount of analyte
- Mean – Accepted true value with confidence interval

The % recovery was calculated using the formula,

$$\%\text{Recovery} = \frac{(a+b)-a}{b \times 100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

Acceptance criteria:

- Individual and mean % recovery at each level should be 98.0% to 102.0%.

5) Precision:

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between the series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.

Method:

- Determination of % relative standard deviation (RSD) of response of multiple aliquots.

Recommendation:

a) Repeatability (Same operating condition over short interval of time):

Minimum of nine determinations

- Low concentration of range × 3 replicates
- Medium concentration of range × 3 replicates
- High concentration of range × 3 replicates

(Or)

- At target concentration × 6 determinations

Acceptance Criteria:

RSD for assay of six determinations should not be more than 2.0%.

b) Intermediate precision (within laboratory variation):

- Different Days
- Different Analysts

- Different Equipment etc.

Expression/calculation:

- Standard deviation, % RSD and confidence interval

Acceptance criteria:

- RSD for assay of six determinations should not be more than 2.0%.
- Difference between the mean assay value obtained in the intermediate precision study and method precision study should not be more than 2.0% absolute.

6) Detection Limit:

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample, which can be detected but not necessarily quantitated under stated experimental conditions.

Method:

1. By visual evaluation
2. Based on S/N ratio
    - Applicable to procedure, which exhibit baseline noise.
    - Actual lowest concentration of analyte detected in compared with blank response
3. Based on S.D. of response and slope

$$LOD = 3.3\ \sigma/s$$

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Expression/calculation:

- If based on visual examination or S/N ratio – relevant chromatogram is to be presented.
- If by calculation/extrapolation – estimate is validated by analysis of suitable no. of samples known to be near or prepared at detection limit.

Acceptance criteria:

- S/N ratio > 3 or 2:1; not specified in other cases.

7) Quantitation Limit:

The quantitation limit of an individual analytical procedure is defined as the lowest amount of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy.

Method:

1. By visual evaluation
2. Based on S/N ratio
    - Applicable to procedure, which exhibit baseline noise.
    - Actual lowest concentration of analyte detected in compared with blank response
3. Based on S.D. of response and slope

$$LOQ = 10\ \sigma/s$$

s = Slope of calibration curve

σ = S.D. of response; can be obtained by

- Standard deviation of blank response
- Residual standard deviation of the regression line
- Standard deviation of the y-intercept of the regression line
- $S_{y/x}$ i.e. standard error of estimate

Recommendation:

- Limit should be validated by analysis of suitable no. of samples known to be near or prepared at quantitation limit.

Expression/calculation:

- Limits of quantitation and method used for determining should be presented.
- Expresses as analyte concentration.

Acceptance criteria:

- S/N ratio > 10:1; not specified in other cases.

8) Robustness:

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Method:

It should show the reliability of an analysis with respect to deliberate variations in method parameters.

In case of liquid chromatography, examples of typical variations are

- Influence of variations of pH in a mobile phase,
- Influence of variations in mobile phase composition,
- Different columns (different lots and/or suppliers),
- Temperature,
- Flow rate.

Recommendation:

- Robustness should be considered early in the development of a method.
- If the results of a method or other measurements are susceptible to variations in method parameters, these parameters should be adequately controlled and a precautionary statement included in the method documentation.

Expression/calculation:

- Effect of these changed parameters on system suitability parameters.

Acceptance criteria:

- System suitability criteria should meet as per test procedure.
- The difference between assay value of sample analyzed as per test procedure and analyzed by applying proposed changes should not be more than 2.0% absolute.

9) Ruggedness:

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by analysis of the same samples under a variety of conditions.

Method:

- Analysis of aliquots of homogenous lots in different laboratories by different analysts under different operational and environmental conditions.

Expression/calculation:

- % RSD

Note: In the guideline on definitions and terminology, the ICH did not address ruggedness specifically. This apparent omission is really a matter of semantics, however, as ICH chose instead to cover the topic of ruggedness as part of precision, as discussed previously.

10) Solution stability:

Prepare standard and sample as per test procedure and determine initial assay value. Store the standard and sample preparation up to 48 hours at room temperature. Determine the assay of sample preparation after 24 hours and 48 hours storage against freshly prepared standard and determine % response of standard preparation after 24 hours and 48 hours storage against initial standard response. The assay value of sample and % response of standard calculated after 24 hours and 48 hours storage should be compared with the initial value and recorded.

If the stability of solution fails to the acceptance criteria at 24 hour interval at room temperature, repeat the experiment and injecting after standing for 2, 4, 8, 12, and 18 hours at room temperature.

If the stability of solution is found to be less than 24 hours at room temperature, then establish the solution stability at 5°C±3°C as per the above procedure.

Calculation: Calculate results as follows:

Standard preparation stability: Calculate the % response of the Standard preparation after specified period using the formula;

$$\% \text{ Response} = \frac{TA}{SA} \times 100$$

Where,

TA = The peak area of standard preparation after standing for specified period.

SA = The initial peak area of standard preparation subjected for solution stability.

Sample preparation stability: Calculate the % Assay of the Sample preparation after specified period as per the test procedure against freshly prepared standard.

Calculate the difference of the result obtained after each interval against initial result.

Acceptance criteria:

- The difference in the response of standard preparation should not be more than ± 2.0% from the initial value at any time interval.
- The absolute difference in the assay value of sample should not be more than ± 2.0% from the initial value at each time point.

11) System Suitability Testing:

The system has to be tested for its suitability for the intended purpose. System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such.

Numerous approaches may be used to set the limits for system suitability tests. This depends on experience with the method, material available and personal preference. Parameters such as plate count, tailing factors, resolution and reproducibility (% RSD retention time and area for six repetitions) are determined and compared against the specifications set for the method.

Table 10: System Suitability Parameters and their recommended limits

Parameter	Recommendation
Capacity Factor (K')	The peak should be well-resolved from other peaks and the void volume, generally K' > 2
Repeatability	RSD ≤ 1% ; N ≥ 5 is desirable
Relative Retention	Not essential as the resolution is stated.
Resolution(R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipients, degradation product, internal standard, etc.)
Tailing Factor(T)	T ≤ 2
Theoretical Plates(N)	In general should be > 2000.

Table 11: Characteristics to be validated in HPLC

Characteristics	Acceptance Criteria
Accuracy/trueness	Recovery 98-102% (individual)
Precision	RSD < 2%
Repeatability	RSD < 2%
Intermediate Precision	RSD < 2%
Specificity / Selectivity	No interference
Detection Limit	S/N > 2 or 3
Quantitation Limit	S/N > 10
Linearity	Correlation coefficient $r^2 > 0.999$
Range	80 –120 %
Stability	> 24 h or >12 h

2.1 LITERATURE SURVEY OF DROTAVERINE HYDROCHLORIDE

Sr No	Method	Description	Reference
1.	Application of UV-spectrophotometric method for estimation of Drotaverine hydrochloride in bulk & tablets.	Detection : 354 nm Linearity range : 4 - 24 µg/ml	51
2	Spectrofluorometric determination of Drotaverine hydrochloride in pharmaceutical preparations.	Fluorescence of the DRT measured in 0.1 M H_2SO_4 Emission: 465 nm Excitation: 295 nm	52
3	A new method for high-performance liquid chromatographic determination of Drotaverine in plasma.	Plasma samples : pH 1.5 Extration with chloroform Stationary Phase : SiO_2 Mobile Phase : n-heptane-dichloromelhane-diethylamine (50:25:2) Detection with variable-wavelength UV detector set at 302 nm	53
4	Modified high-performance liquid chromatographic method for analysis of Drotaverine in human plasma.	Internal standard: Papaverine LOD : 50 ng ml−1	54
5	High-performance liquid chromatographic method for the determination of	Stationary Phase : C18-column Mobile Phase :0.02 M sodium dihydrogen	55

	Drotaverine in human plasma and urine.	phosphate—methanol (30:70, v/v) containing perchlorate ion at pH 3.2 Detection : 254 nm LOD : 6 ng/ml	
6	An ion-selective electrode for quantitative analysis of therapeutic formulations of drotaverine hydrochloride.	By using Ion Selective Electrode(ISE). By Direct potentiometry and potentiometric depositional titration. ISE reacted reversibly to changes in the potential determining ion concentration over the range 5 × 10− 2 to 7.9 × 10− 6 M, the detection limit was (4.3 ± 0.2) × 10− 6 M electrode function slope was 58 ± 2 mV/Pc.	56
7	Application of new membrane selective electrodes for the determination of Drotaverine hydrochloride in tablets and plasma.	Poly Vinyl Chloride (PVC) membrane sensors are used. PVC based on the use of the ion association complexes of drotaverine cation with sodium phosphotungestate (Dro-PTA) or ammonium reineckate (Dro-R) counter anions as ion exchange sites in the PVC matrix. Linearity Range : 10^{-5}-10^{-2}M	57
8	Cathodic adsorptive stripping voltammetry of Drotaverine hydrochloride and its determination in tablets and human urine by differential pulse voltammetry.	By using Hanging mercury drop electrode. Linearity Range : 21.70–257.34 ng/ml LOD : 3.15 ng/ml LOQ : 10.50 ng/ml	58
9	Potentiometric flow injection analysis of Drotaverine hydrochloride in pharmaceutical preparations.	By using five poly (vinyl chloride) (PVC) membrane electrodes. The membranes of these electrodes consist of drotaverinium-silicotungstate (Dv-ST), silicomolybdate (Dv-SM), phosphotungstate (Dv-PT), phosphomolybdate (Dv-PM), or	59

		tetraphenylborate (Dv-TPB) ion associations dispersed in PVC matrix with dibutyl phthalate plasticizer. Electrodes showed near-Nernstian response in range of 2.0×10^{-6} to 1.0×10^{-2} M DRT. LOD – 0.87 µg/ml	
10	Spectrophotometric methods for simultaneous estimation of Nimesulide and Drotaverine.	**1st method**:Q-absorbance equation **2nd method**: Simultaneous equation method **3rd method**: Multicomponent mode absorbance. Linearity : 5 -30 µg/ml For both DRT & NIM	60
11	Simultaneous determination of Drotaverine hydrochloride and Aceclofenac in tablet dosage form by spectrophotometry.	**1st method** is Absorbance Ratio Method. **2nd** is Simultaneous equation method (Vierodt's method). **3rd method** is based on first order derivative spectroscopy. Linearity : 10-50µg/ml for both drugs	61
12	Simultaneous determination of Nifuroxazide and Drotaverine hydrochloride in pharmaceutical preparations by bivariate and multivariate spectral analysis.	**Method A**: Bivariate spectrophotometric analysis. **Method B**: Multivariate spectral analysis. Linearity : 2-10µg/ml	62
13	Spectrophotometric determination of Pipazethate HCl, Dextromethorphan HBr and Drotaverine HCl in their pharmaceutical	Involves use of chromotrope 2B & chromotrope 2R. Method consists of extracting the formed ion-associates into chloroform in the case of Pipazethate HCl and Dextromethorphan HBr	63

	preparations.	or into methylene chloride in the case of Drotaverine HCl. Ion-associates exhibit absorption maxima at 528, 540 and 532 nm with chromotrope 2B and at 526, 517 and 522 nm with chromotrope 2R for pipazethate HCl, dextromethorphan HBr and drotaverine HCl, respectively. Linearity : For PPZ 4.36–52.32 μg mL^{-1} 3.7–48.15 μg mL^{-1} for DEX 4.34–60.76 μg mL^{-1} for DRT	
14	Selective differential spectrophotometric methods for determination of Niclosamide and Drotaverine hydrochloride.	**Method A**: Differential (ΔA) spectroscopy. **Method B**: second derivative (ΔD2) spectroscopy. **Method C:** Third derivative (ΔD3) spectroscopy.	64
15	High performance liquid chromatographic estimation of Drotaverine hydrochloride and Mefenamic acid in human plasma.	Extraction: Dichloromethane, and isopropyl alcohol in the ratio 80:20 (v/v). Stationary Phase: Thermo BDS Hypersil C8 (25.0 cm×4.6 mm, 5 μm particle size). Mobile Phase: Acetonitrile and Ammonium acetate buffer (20 mM, pH 3.5 ± 0.05 adjusted with 85% phosphoric acid) in a ratio of 55: 45 (v/v). FR : 1 mL min-1 UV detection at 230 nm Diclofenac sodium (internal standard) 32-960 ng/mL LOD 11 & LOQ 32 ng/ml for DRT 100-3000 ng/mL LOD 33 & LOQ 100 ng/ml	65

		for MEF	
16	Development and validation of an RP-HPLC method for simultaneous analysis of Drotaverine and Omeprazole in a tablet dosage form.	Stationary Phase :C18 column Mobile Phase : 60:40 (v/v) Methanol & Ammonium acetate (0.1 M , pH 5, adjusted with OPA) FR : 1.5 mL min^{-1} UV detection :319 nm Linearity : 5-40 ng/ml for DRT & 5-50 ng/ml for OMP LOD: 16.2 and 4.8 ng mL^{-1} & LOQ 49.0 and 14.5 ng mL^{-1} for DRT and OMP	66
17	Determination of Nifuroxazide and Drotaverine hydrochloride in pharmaceutical preparations by three independent analytical methods.	**Method A:** Spectrophotometry **Method B:** Spectrodensitometry method Stationary phase: silicagel Mobile phase: choloform : acetone : methanol: gl HAC (6:3:0.9:0.1) Detection : 365 nm **Method C**:RP HPLC Mobile phase : ACN : Water (40:60) pH- 2.55 with OPA FR :1 ml/min Detection : 285 nm Pentoxifyline as internal standard	67
18	Application of derivative, derivative ratio, and multivariate spectral analysis and thin- layer chomatography-densitometry for determination of a ternary	**Method A**:First (D1) and third (D3) derivative spectrophotometry **Method B** : Simultaneous use of the first derivative of the ratio spectra **Method C** :Thin-layer chromatography	68

	mixture containing Drotaverine hydrochloride, Caffeine, and Paracetamol.	Stationary Phase :silica gel Mobile phase :Ethyl acetate-Chloroform-Methanol (16 + 3 + 1, v/v/v)	
19	Spectrophotometric and spectrodensitometric determination of Paracetamol and Drotaverine HCl in combination.	**Method A** :TLC densitometric method Mobile Phase : Ethyl acetate:methanol:NH_3 (100:1:5 v/v/v) **Method B**: Spectrophotometric method **Method C**: First derivative spectroscopic **Method D**: Vierordt's method	69
20	A comparative study on various spectrometries with thin layer chromatography for simultaneous analysis of Drotaverine and Nifuroxazide in capsules.	Three spectrophotometric methods **Method A** :Vierordt's method **Method B** :Ratio spectra derivative **Method C**: Derivative spectroscopy **Method D** :Thin layer chromatography (TLC)-UV densitometric method Mobile phase :Ethyl acetate : methanol : ammonia 33% (10 : 1 : 0.1 v/v/v) Detection: Densitometrical area were measured at 308 and 287 nm	70
21	Quantitative estimation of benzylisoquinoline derivatives by coulometric titration.	Drotaverine HCl by coulometric titration with biamperometric indication of the end point.	71
22	Spectrophotometric determination of some anti-tussive and anti-spasmodic drugs through ion-pair complex formation with thiocyanate and cobalt (II)	Drugs: Dextromethorphan hydrobromide, Pipazethate hydrochloride, Drotaverine and Trimebutine maleate The reaction of cobalt(II)–thiocyanate complex extraction : n-butnol–dichloromethane solvent mixture (3.5:6.5) Detection : 625 nm	72

	or molybdenum (V).	Molybdenum(V)–thiocyanate ions complex extraction: methylene chloride 467 or 470 nm for (DEX and Pip) or (DRT and TM), respectively	
23	Stability-indicating HPTLC method for simultaneous estimation of Drotaverine and Nimesulide in pharmaceutical dosage form.	Stationary phase: silica gel 60 F254 plate : Mobile phase: Cyclohexane: Methanol: Ethyl acetate (6: 2: 2 v/v/v) Densitometric scanning: 295 nm. Rf values: 0.15 for DRT and 0.53 for NIM. Linearity: 100-600ng/band for DRT and 200-700ng/band NIM	73

2.2 DEGRADATION of DRT HCl [(74)]:

- Literature survey revealed that in the neutral and basic conditions, the DRT HCl is hydrolysed to **DROTAVERALDINE**.
- While in the presence of light, it decomposes to **PERPARINE** and finally to **PERPARALDINE** by dehydrogenation.
- It also interacts with tabelting vehicles like Magnesium Stearate and some glidents during tabelting which cause hydrolysis of API as in basic condition.

Aim of Present Work

Drotaverine is an antispasmodic drug. It is selective phosphodiesterase IV inhibitor. Drotaverine is found be nearly 80% effective in treating renal colic. It has also been used to accelerate labor.

Based on the literature review, it was found that a number of analytical studies involving measurement of DRT concentration by HPLC with UV detector, thin layer chromatography, spectrophotometry, spectrofluorymetry, coulometry titration, potentiometry, voltametry for individual/simultaneous estimation and/or separation in formulations/biological specimen have been developed. No stability indicating LC method has been reported in the literature so far for the determination of the drug in the presence of its degradation products from its pharmaceutical formulations.

So, the aim of the present work was the Development and Validation of stability indicating HPLC with method for the estimation of Drotaverine HCl in presence of its degraded products formed under different stress conditions in pharmaceutical dosage form. The objective of the study was to establish the degradation pathway and intrinsic stability of molecule.

The proposed method shall be used for the quantification of Drotaverine HCl in routine quality control analysis in pharmaceutical dosage forms.

The proposed method was validated for accuracy, precision, and repeatability as per ICH guideline.

Drotaverine HCl :

Gift sample was received from Troikaa pharmaceutical pvt ltd.

Assay: 98-101 %

4.1. UV SPECTRUM:

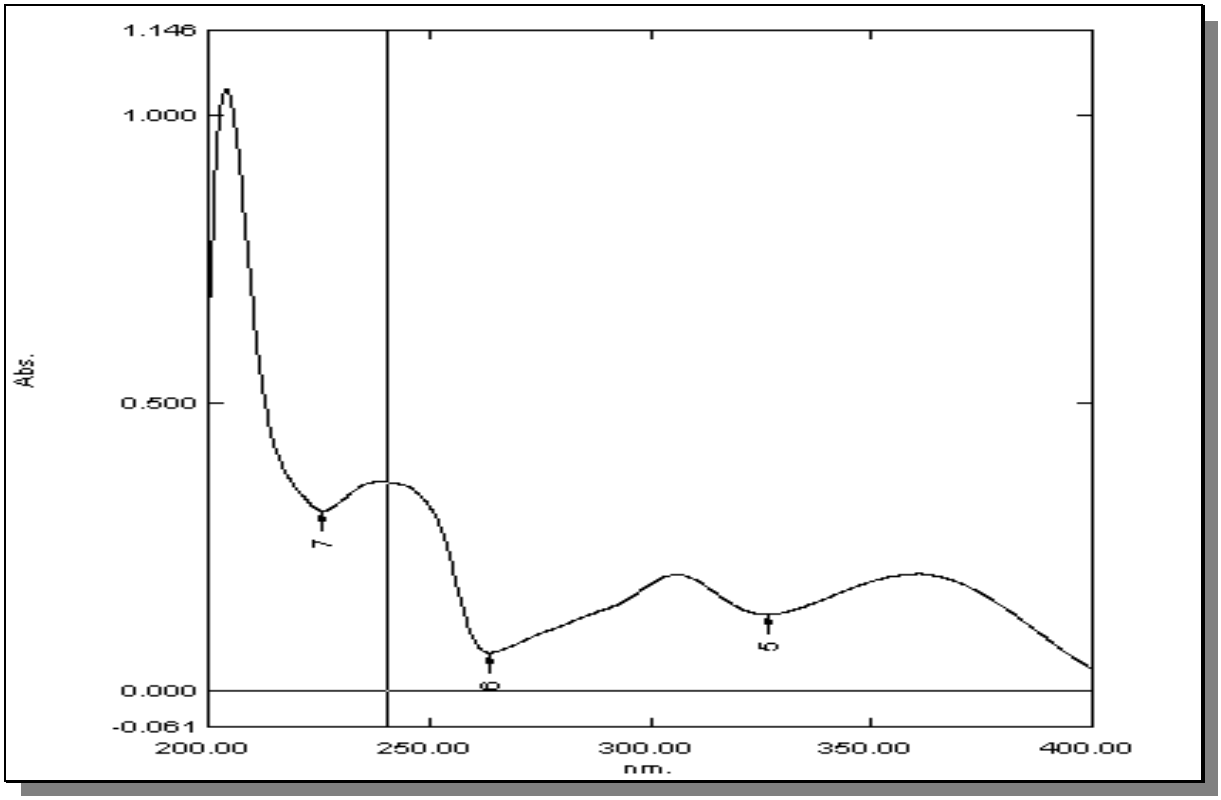


Figure 9: UV spectrum of 10 ppm Drotaverine HCl in acetonitrile

Table 12: Maxima & Minima of DRT UV spectrum

Maxima reported	Maxima obtained	Minima reported	Minima observed
241 nm	240 nm	223 nm	226 nm
302 nm	306.2 nm	262.5 nm	264 nm
354.5 nm	360.2 nm	321 nm	326.2 nm

4.2. Raman spectra of Drotaverine HCl:

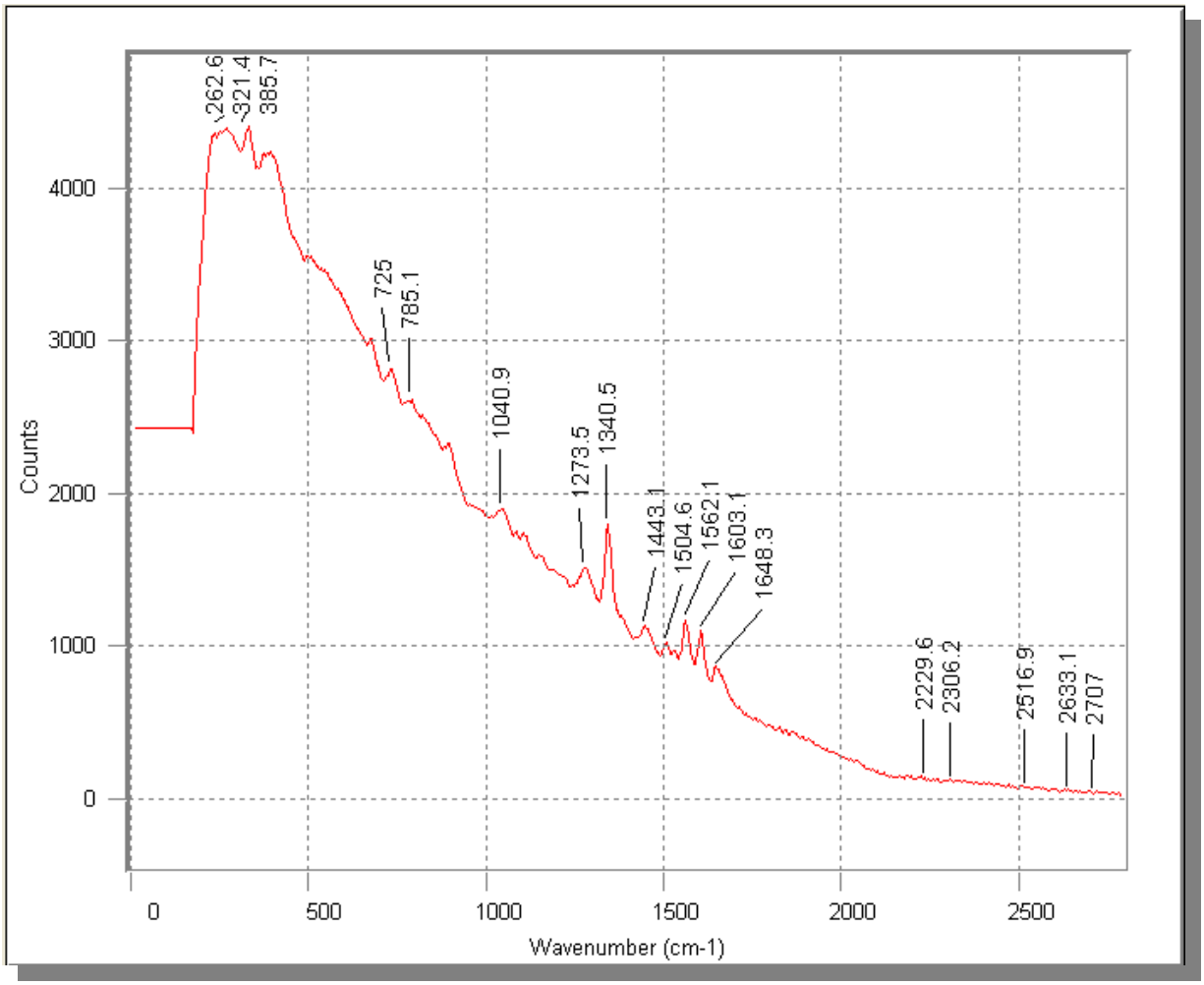


Figure 10: Raman spectra of DRT

Table 13: Interpretation of Raman Spectra [75]

Frequency reported (cm^{-1})	Frequency observed (cm^{-1})	Functional Group
1251-1270	1272	Di alkyl ethylene
1020-1060	1040.9	Ortho di substituted benzene
1550-1630	1504	Benzene derivative
830-930	Not observed	C-O-C & C-N-C

4.3. MELTING POINT:

Table 14: Melting point of DRT HCl

Reported melting Point	Observed melting Point
208-212 °C	205-210°C

STABILITY INDICATING RP-HPLC ESTIMATION OF DROTAVERINE HYDROCHLORIDE IN PHARMACEUTICAL FORMULATIONS

5.1. MATERIAL & INSTRUMENTATION:

5.1.1. Instrumentation

a) Jasco HPLC:

- Model: series 200
- Manufacturer: Jasco Japan
- Pump: Jasco PU 2080 plus
- Mixer: Jasco MX 2080-3”
- Injector: Reodyne valve with 20 µL fixed loop
- Detector: Jasco UV 2075 plus
- Software: Borwin

b) Column:

- Name: Inertsil^{R} ODS 3V, GL Science Inc.
- Manufacturer: Japan
- Particle size: 5 µ
- Length: 150 mm
- Diameter : 4.6 mm

c) UV spectrometer:

- Model: 2450
- Manufacture : Shimadzu Japan

d) Digital pH meter:

- Manufacturer : Analabs scientific India

e) Ultra sonic cleaner

- D compact 936

f) Sohxlet apparatus:

- Manufacturer: Durga scientific Pvt Ltd.

g) Hot air oven:

- Marketed by: EIE Instrument Pvt Ltd.

h) Digital balance citizen:

- Model: Cx 220

i) Raman spectrometer

j) Rocker vaccum pump.

5.1.2. Reagents :

- ***ACN,*** HPLC and spectroscopic grade (Merck laboratory)
- Milipore HPLC ***water***
- Potassium di hydrogen phosphate, KH_2PO_4 : AR grade (CDH laboratory)
- 30 % w/v H_2O_2 AR grade (SD fine chemicals)
- ***Tri ethyl amine***, AR grade (SD fine chemicals)
- **NaOH** pellets, AR grade (CDH laboratory)
- Concentrated ***HCl*** (35.4 % v/v) AR grade (SD fine chemicals)

5.1.3. Apparatus :

- Round bottom flask (250 ml)
- Volumetric flask (10 ml , 25 ml , 50 ml and 100 ml)
- Beaker (100 & 250 ml)
- Membrane filter 0.22 µ (Millpores Ltd)
- Syringe filter 0.45 µ (Millpores Ltd)
- Pipettes (1 ml ,2 ml , 5 ml, 10 ml)
- Testtubes

5.2. SELECTION OF THE WAVELENGTH :

25 mg of the API was weighed accurately and diluted up to 25 ml with diluent containing ACN: water (50:50, V/V)

↓

It was diluted further to obtain 10 µg/ml

↓

Scan from the 200 to 400 nm

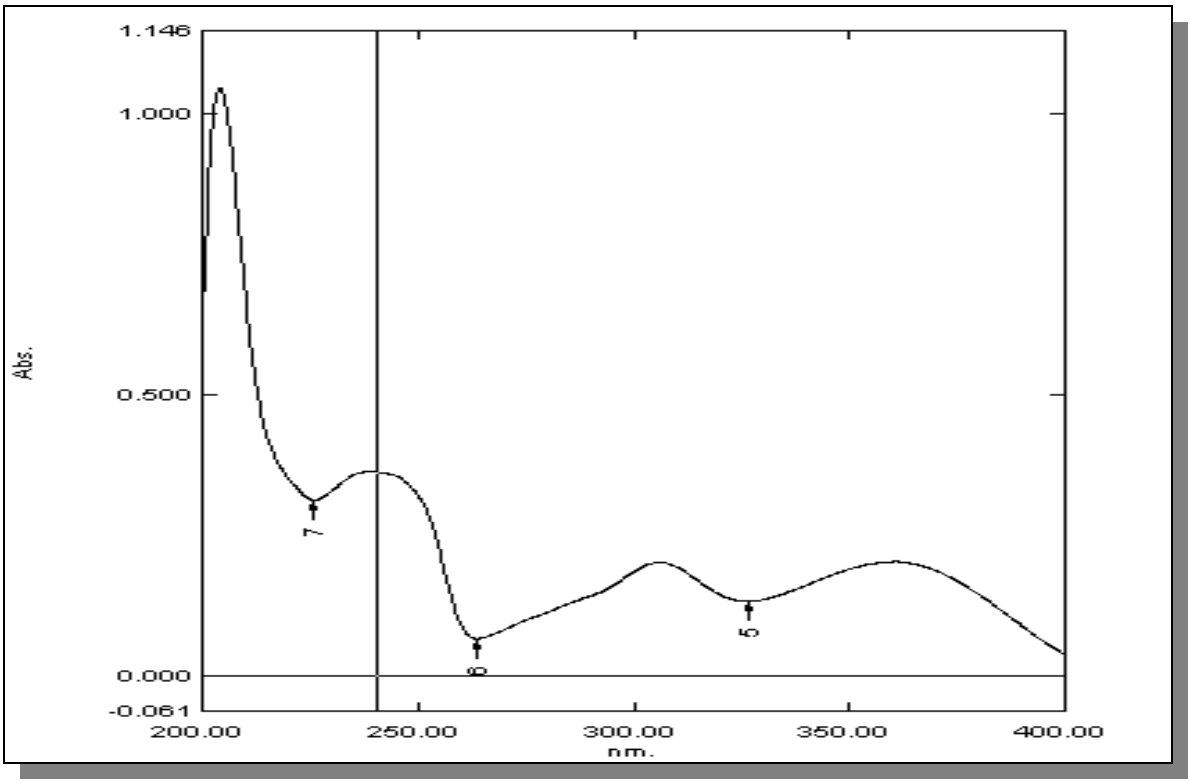


Figure 9: UV spectrum of Drotaverine HCl (10 µg/ml)

- **Selected wavelength : 240 nm**

5.3. MOBILE PHASE OPTIMISATION:

Table 15: Mobile Phase optimisation

Mobile phase composition	Flow rate	Observation	Conclusion
Methanol : Water (50:50, v/v)	1 ml/min	No elution of Drug **Chromatogram: 1**	Mobile phase not selected
ACN: Water (85:15, v/v)	1 ml/min	Rt =7.1 min **Chromatogram:2**	Mobile phase not selected
ACN: KH_2PO_4 (0.01M) Buffer (60:40, v/v) pH 4 (with OPA)	1 ml/min	**Rt= 2.8** **SSP not satisfied**	Mobile phase not selected
ACN: KH_2PO_4 (0.01M) Buffer (43:57, v/v) ; 0.1% TEA solution pH 4 (with OPA)	0.8 ml/min	**Rt= 4.4** **Asymmetry 1.3** **Plates : 2512** **Chromatogram:3**	Mobile phase selected

FINALIZATION OF MOBILE PHASE:

- **ACN : KH_2PO_4 (0.01M) with 0.1% TEA (pH-4, with dilute OPA)**
- **Composition** : 43: 57 % v/v
- **Flow rate**: 0.8 ml/min
- **Wavelength** : 240 nm
- **Peak report:**
    - Retention time: 4.4
    - Asymmetry: 1.29
    - Theoretical plates : 2512

5.4. CHROMATOGRAPHIC CONDITIONS:

A reverse phase C18 column equilibrated with mobile phase acetonitrile: 0.01 M potassium dihydrogen phosphate (43:57, v/v; pH 4.0) was used on isocratic mode. Mobile phase flow rate was maintained at 0.8 ml/min and effluents were monitored at 240 nm. The sample was injected using a 20 µL fixed loop, and the total run time was 25 min.

5.5. PREPARATION OF MOBILE PHASE :

1. ***Mobile phase A*** - ACN: HPLC grade acetonitrile was taken in mobile phase reservoir and degassed for 10 min with Ultra sonic cleaner for degassing.

2. ***Water***: Milipore HPLC grade water was filtered twice with 0.22 µ membrane filter and sonicated for 10 mins for degassing.

3. ***Mobile phase B***-Phosphate buffer: 0.3402 gm of KH_2PO_4 was weighed accurately and dissolved in 250 ml HPLC water to obtain 0.01M. pH was adjusted to 4 with diluted ortho-phosphoric acid. Solution was filtered twice with 0.22 µ membrane filter and sonicated for 10 min for degassing.

5.6. STANDARD SOLUTION PREPARATION:

25 mg of Drotaverine HCl was weighed accurately and dissolved in 25 ml of methanol (AR grade) to obtain 1 mg/ml of stock solution. From the stock solution, 0.1 ml was taken and diluted with ACN: water (50:50, v/v) to obtain working standard of 10 µg/ml.

5.7. CALIBRATION CURVE FOR DRT:

Appropriate aliquots of stock solution were taken in different 10 ml volumetric flasks and diluted up to the mark with diluents containing acetonitrile: water (50:50, v/v) to obtain final concentrations of 0.5, 1, 2, 4, 8, 16, 32 µg/ml of DRT, respectively. The solutions were injected in replicate using a 20 µL fixed loop system and chromatograms were recorded. Calibration curve was constructed by plotting average peak area versus concentrations and regression equation was computed for DRT.

5.8. FORMULATIONS CONTAINING DROTAVERINE HCl:

- Drotaverine HCl injection containing 20 mg/ml of Drotaverine HCl of **Troikaa Pharmaceutical Pvt Ltd.(Formulation A)**

- Drotin injection containing 20 mg/ml of Drotaverine HCl of **Martyn and Haris Pharmaceutical Pvt Ltd.(Formulation B)**

- Drotikind injection containing 20 mg/ml of Drotaverine HCl of **Lifestar Pharma Pvt Ltd.(Formulation C)**

- Verine injection containing 20 mg/ml of Drotaverine HCl of **Corona Remedies Pvt Ltd.(Formulation D)**

5.9. ASSAY FOR THE MARKETED FORMULATIONS:

1.25 ml of each formulation was taken and dissolved in 25 ml of methanol (AR grade) to obtain 1 mg/ml of stock solution. From the this stock solution, 0.1 ml was taken and diluted with ACN:water (50:50, v/v) to obtain working standard of 10 µg/ml and injected under above chromatographic conditions and peak area was measured & compared with area of Drotaverine HCl.

Chromatograms: 4,5,6,7 for Formulation A, B, C, D respectively.

5.10. FORCED DEGRADATION STUDY:

Forced degradation study procedure for API:

25 mg of the drotaverine HCl was weighed and subjected to forced degradation in ordered to get less than 30 % degradation.

API was subjected to the forced degradation for acid and base induced hydrolysis; dry (thermal) and water degradation; chemical oxidation and photo degradation.

Forced degradation study procedure for formulations:

1.25 ml from each injection containing drotaverine HCl was taken and subjected similar procedure of forced degradation as in case of the API. Chromatograms obtained for the formulations were compared with chromatograms of API.

5.10.1. BASE DEGRADATION

***Base degradation for API*:**

25 mg of Drotaverine HCl was accurately weighed and transferred to RBF

↓

5 ml of 1 N NaOH was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 1 hr with sohxlet apparatus

↓

Mixture was neutralized with 1 N HCl

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC

Chromatogram base degraded API: 8

Base degradation for the formulations:

1.25 ml of each injection was transferred to RBF

↓

5 ml of 1 N NaOH was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 1 hr with sohxlet apparatus

↓

Mixture was neutralized with 1 N HCl

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatograms were compared with that of the API

Chromatograms base degraded formulations: 9,10,11,12 for A, B, C, D respectively.

5.10.2. ACID DEGRADATION

Acid degradation for API:

25 mg of Drotaverine HCl was accurately weighed and transferred to RBF

↓

5 ml of 1 N HCl was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 4 hr with sohxlet apparatus

↓

Mixture was neutralized with 1 N NaOH

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC

Chromatogram of Acid degraded API: 13

Acid degradation for the formulations:

1.25 ml of each injection was transferred to RBF

↓

5 ml of 1 N HCl was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 1 hr with sohxlet apparatus

↓

Mixture was neutralized with 1 N NaOH

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatograms were compared with that of the API.

Chromatograms of acid degraded formulations: 14,15,16,17 for A, B, C, D respectively.

5.10.3. NEUTRAL DEGRADATION

Neutral degradation of API:

25 mg of Drotaverine HCl was accurately weighed and transferred to RBF

↓

5 ml of milipore water was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 4 hr with sohxlet apparatus

↓

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC

Chromatogram of API subjected to Neutral degradation: 18

Neutral degradation of formulations:

1.25 ml of each injection was transferred to RBF

↓

5 ml of milipore water was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 4 hr with sohxlet apparatus

↓

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatograms were compared with that of the API

Chromatograms of Formulations subjected to Neutral degradation: 19,20,21,22 for A, B, C, D respectively.

5.10.4. CHEMICAL OXIDATION

Stress condition of Oxidation for API:

25 mg of Drotaverine HCl was accurately weighed and transferred to RBF

↓

5 ml of **3%**w/v of H_2O_2 was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50, v/v) was added

↓

Refluxed at 100°C for 45 mins with sohxlet apparatus

↓

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC

Chromatogram of API subjected to oxidation: 23

Stress condition of Oxidation for formulations:

1.25 ml of each injection was transferred to RBF

↓

5 ml of **3%** w/v of H_2O_2 was added to it

↓

5 ml of diluent containing acetonitrile: water (50:50. v/v) was added

↓

Refluxed for 45 mins with sohxlet apparatus

↓

And diluted up to 25 ml in ordered to get 1 mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatograms were compared with that of the API

Chromatograms of Formulations subjected to chemical oxidation: 24,25,26,27 for A, B, C, D respectively.

5.10.5. THERMAL DEGRADATION

Thermal degradation of API:

100 mg of the API was transferred to the petridish

↓

Petridish was put in to hot air oven

↓

API was kept for 6 hrs at 80 ° C

↓

From it, 25 mg of the degraded sample was taken

&

Dissolved in 25 diluent to get 1mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatogram of API subjected to thermolysis: 34

Thermal degradation of formulations:

Formulation as such were put in to petridish

↓

Petridish was put in to hot air oven

↓

And kept for 6 hrs at 80 ° C

↓

From it, 1.25 ml of the degraded sample was taken

&

Dissolved in 25 diluents to get 1mg/ml stock solution

↓

Stock was diluted to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatograms were compared with that of the API

Chromatograms of Formulations subjected to thermolysis: 34,35,36,37 for A,B,C,D respectively

5.10.6. PHOTO DEGRADATION

Photo degradation of API:

25 of the API was weighed and dissolved in 50 ml DW in 250 ml beaker

i.e. 0.5 mg/ml

↓

Sonicate for 10 mins

Beaker was covered with petridish from both sides

↓

And kept in a tray filled with water in ordered to prevent evaporation

Sample was kept in sun light for 6 hrs

↓

0.2 ml from above solution was diluted up to 10 ml in ordered to get 10 µg/ml

And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatogram of API subjected to photolysis: 28

Photo degradation of formulations:

1.25 ml of each injection dissolved in 50 ml DW in 250 ml beaker

i.e. 0.5 mg/ml

↓

Sonicate for 10 mins

Beakers were covered with petridish from both sides

↓

And kept in a tray filled with water in ordered to prevent evaporation

Sample was kept in sun light for 6 hrs

↓

0.2 ml from above solution was diluted up to 10 ml in ordered to get 10 µg/ml

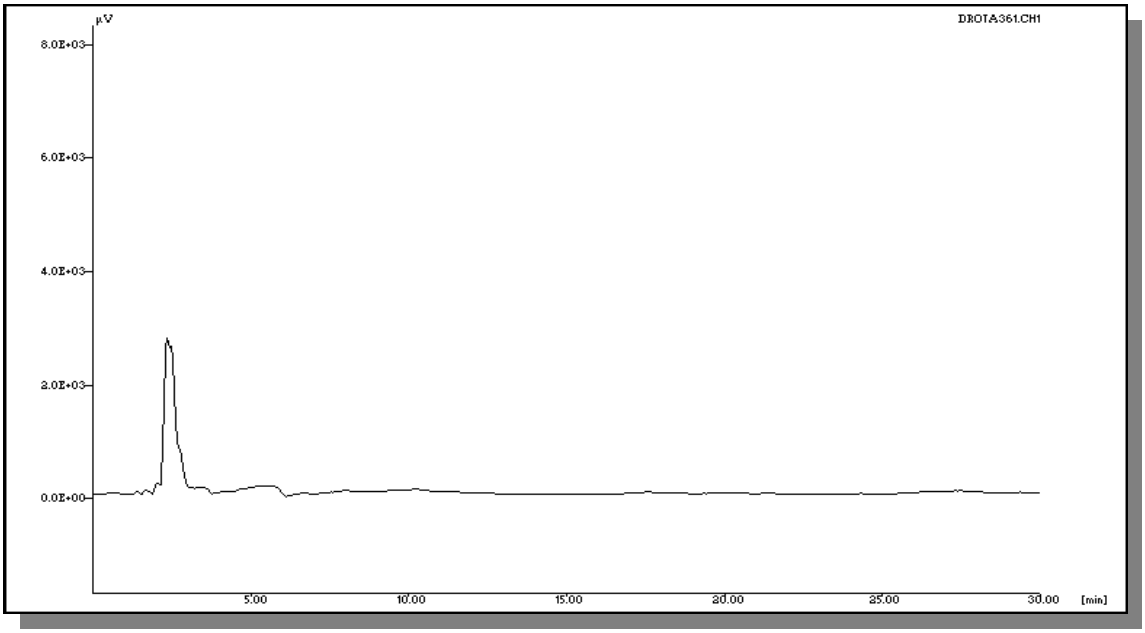
And filtered with syringe filtered (0.45µ) injected in to HPLC system

Chromatograms were compared with that of the API

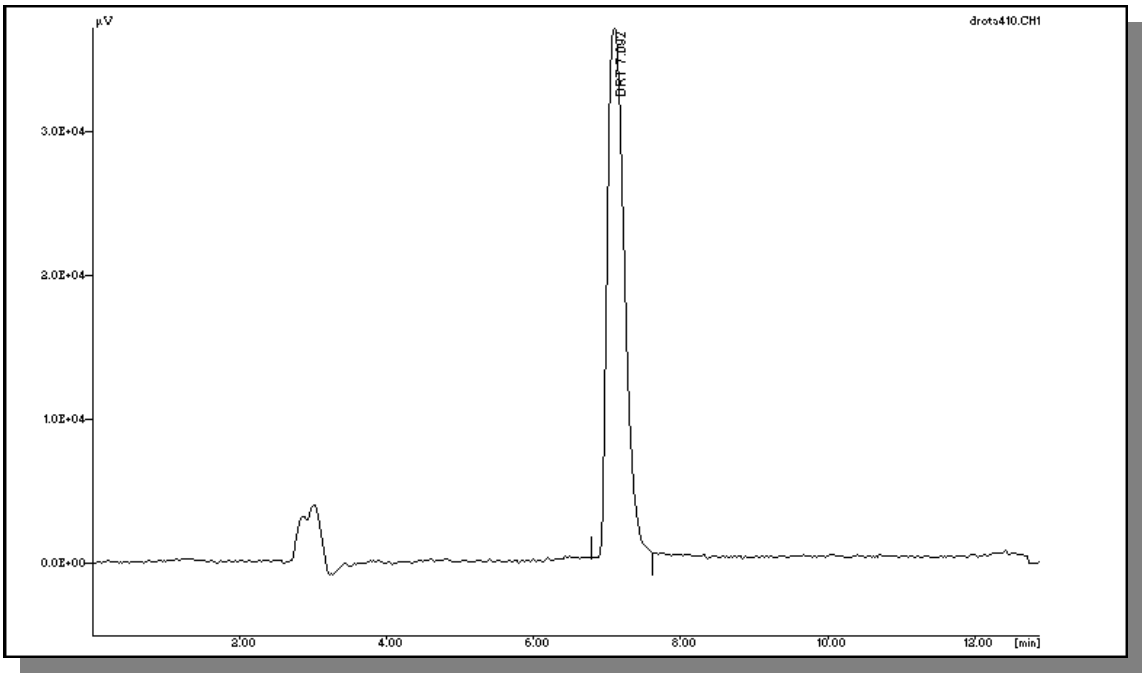
Chromatogram of Formulations subjected to photolysis: 29,30,31,32 for A,B,C,D respectively.

- **Chromatogram** of Blank i.e. ACN:Buffer 50:50 v/v : 38
- **Placebo did not show any interference peak in any degradation.**

Chromatograms of Placebo degradation (of Formulation A only): 39, 40, 41, 42, 43 & 44 subjected to Base, Acid, Thermal, Photo, Neutral & Peroxide degradation respectively.

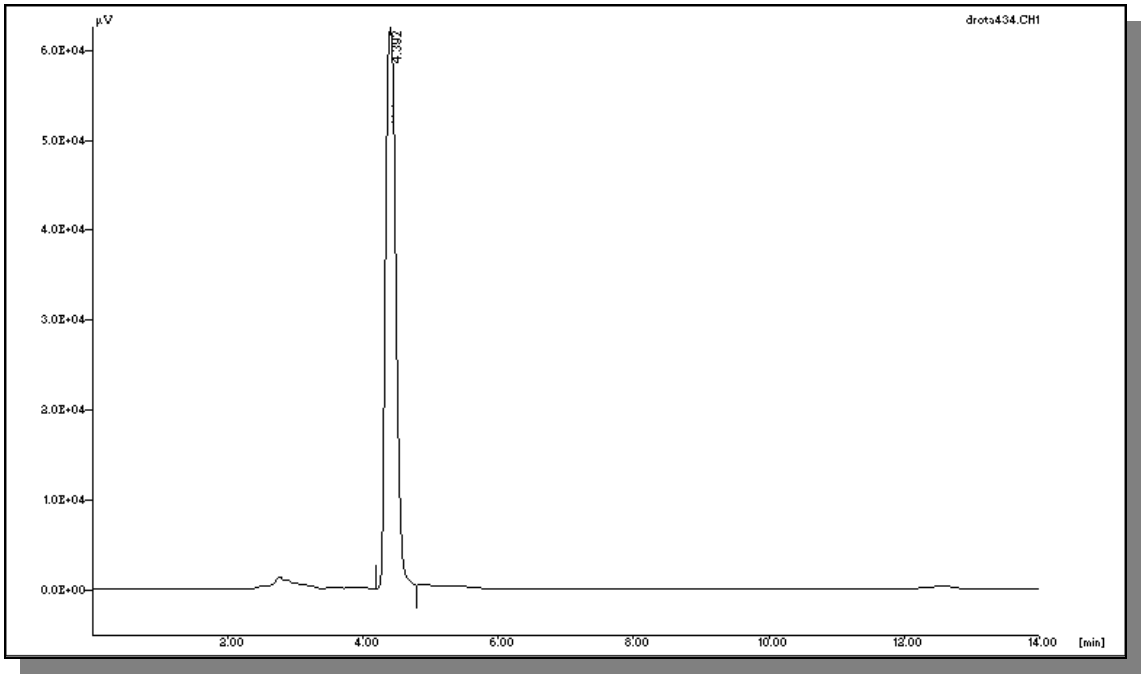


Chromatogram 1: Methanol: Water (50:50, v/v) (no elution of drug)



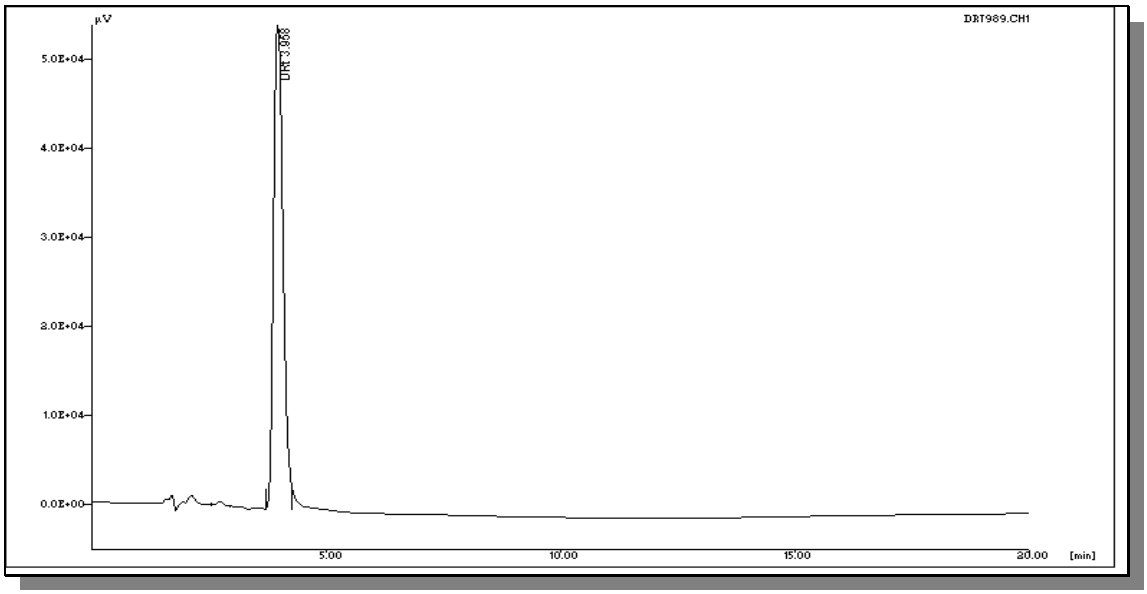
Chromatogram 2: ACN:Water (85:15, v/v)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	7.1	550301	1.48	2107	100	-



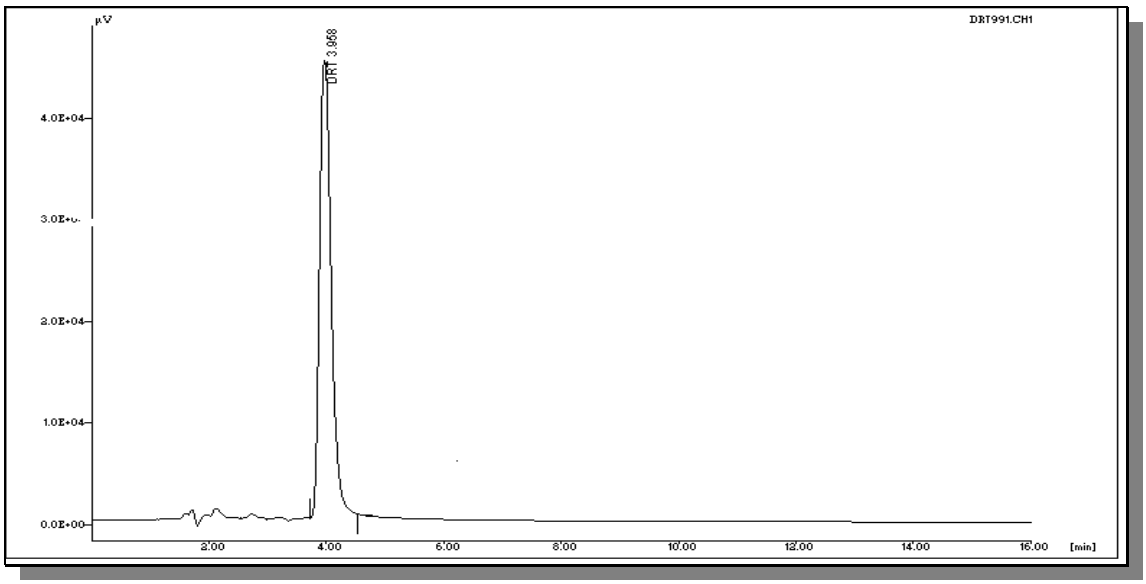
Chromatogram 3: ACN:Buffer(43:57, v/v) FR : 0.8 ml/min

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	4.4	572379	1.29	2512	100	-



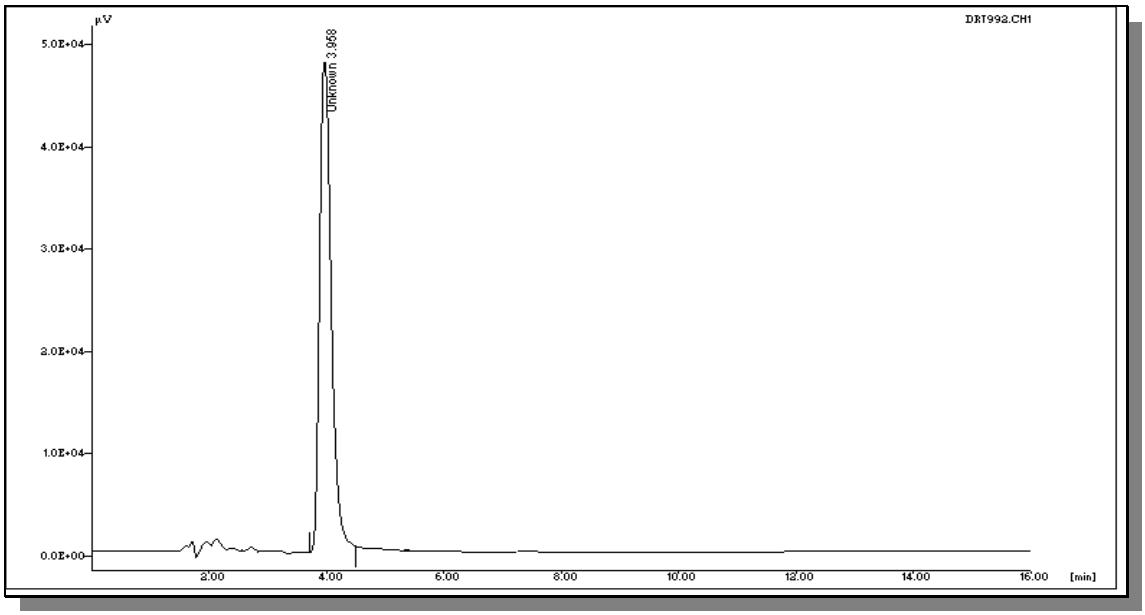
Chromatogram 4: Formulation A 10 µg/ml

Sr No.	Name	Rt	Area	Asymmetry	Plates	%Area	Resolution
1	DRT	3.9	586976	1.21	2506	100	-



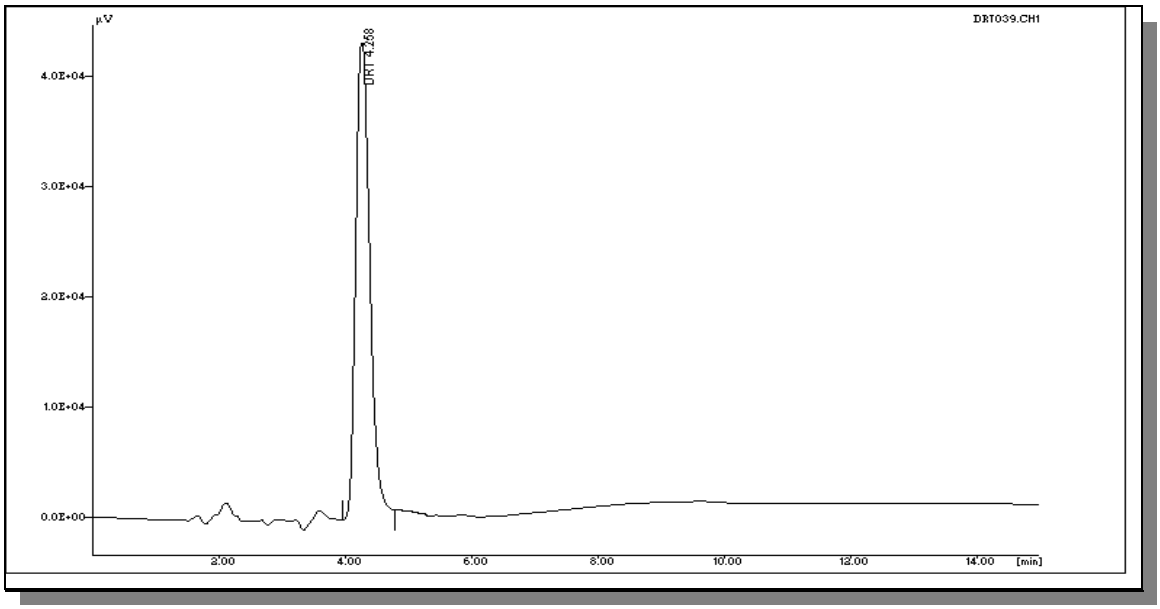
Chromatogram 5: Formulation C 10 µg/ml

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	3.9	581270	1.35	2446	100	-



Chromatogram 6: Formulation B 10 µg/ml

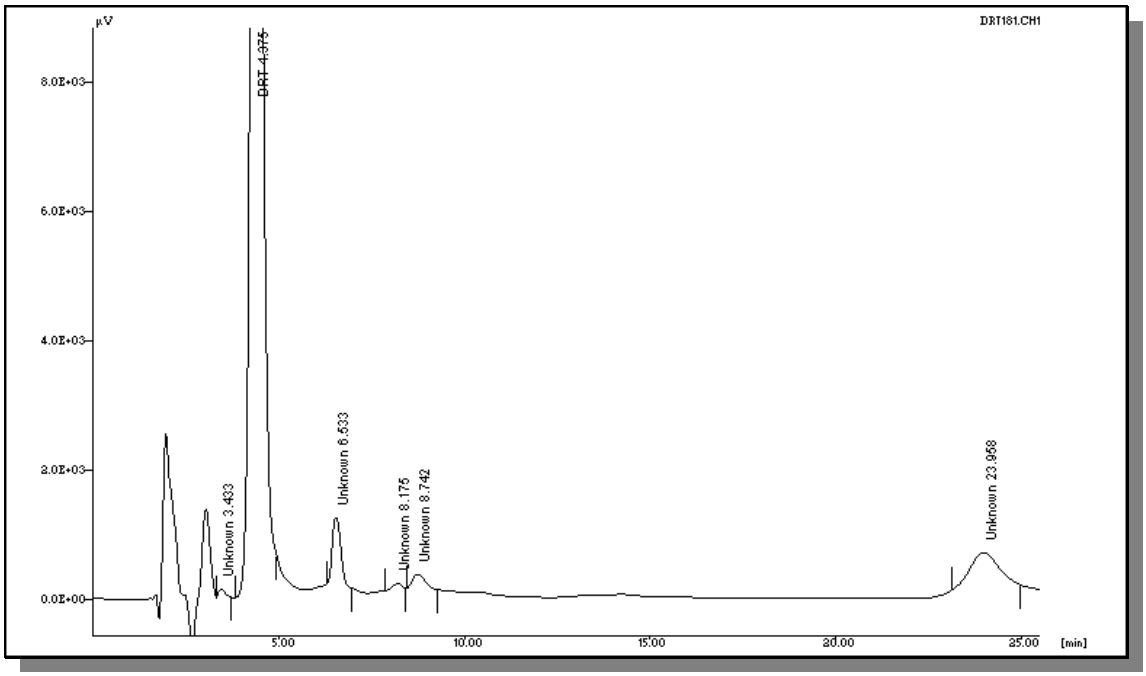
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	3.9	592568	1.36	2472	100	-



Chromatogram 7: Formulation D 10 µg/ml

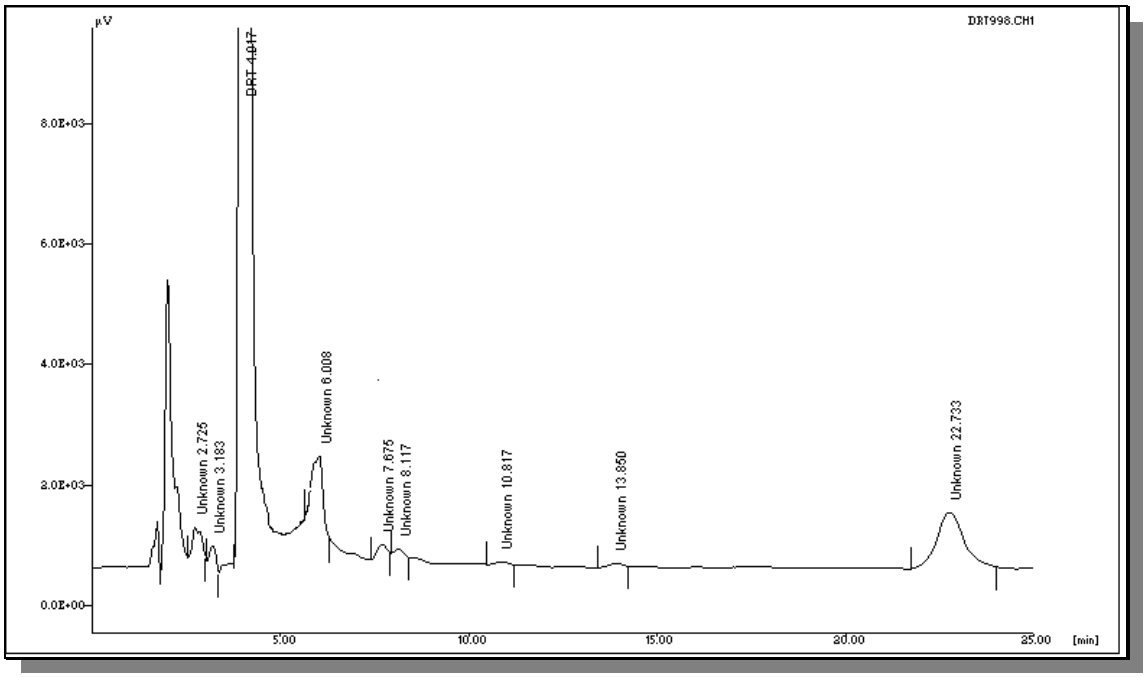
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	4.2	596086	1.27	2216	100	-

Chromatograms of BASE degraded samples:



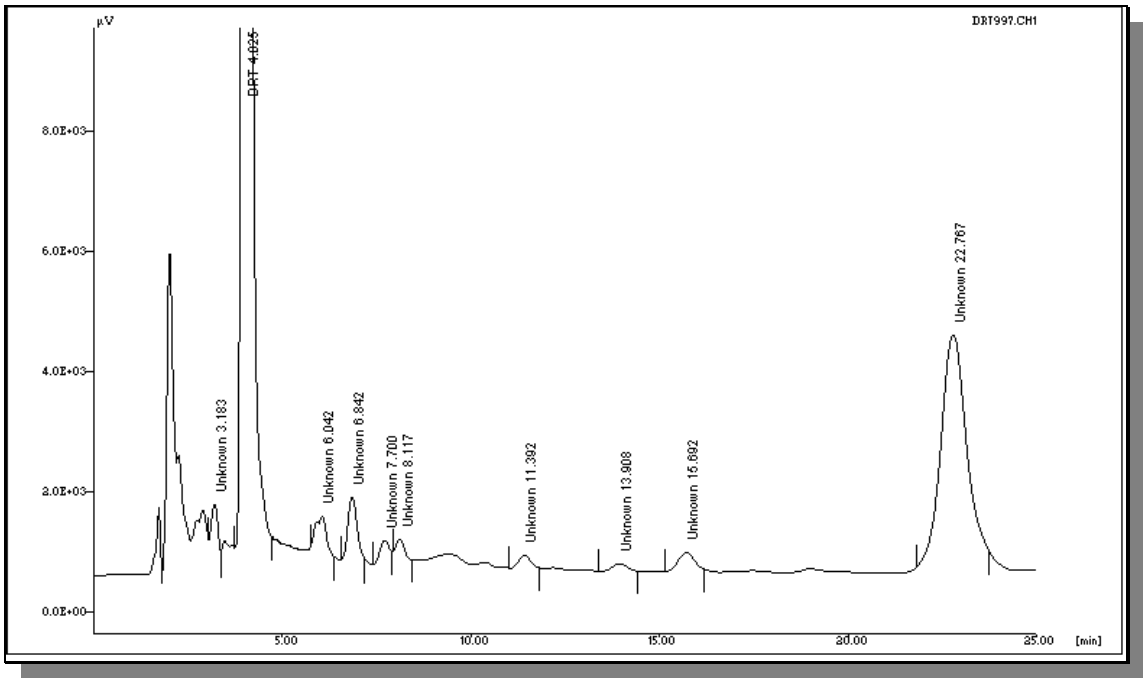
Chromatogram 8: API base degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.4	1526	1.62	2134	0.3	-
2	DRT	4.4	449921	1.14	1817	89.91	2.67
3	Unknown	6.5	14496	1.33	4712	2.9	5.47
4	Unknown	8.2	1351	0.91	5760	0.27	4.05
5	Unknown	8.7	4828	1.33	3466	0.96	1.11
6	Unknown	24	28267	1.08	4785	5.65	15.41



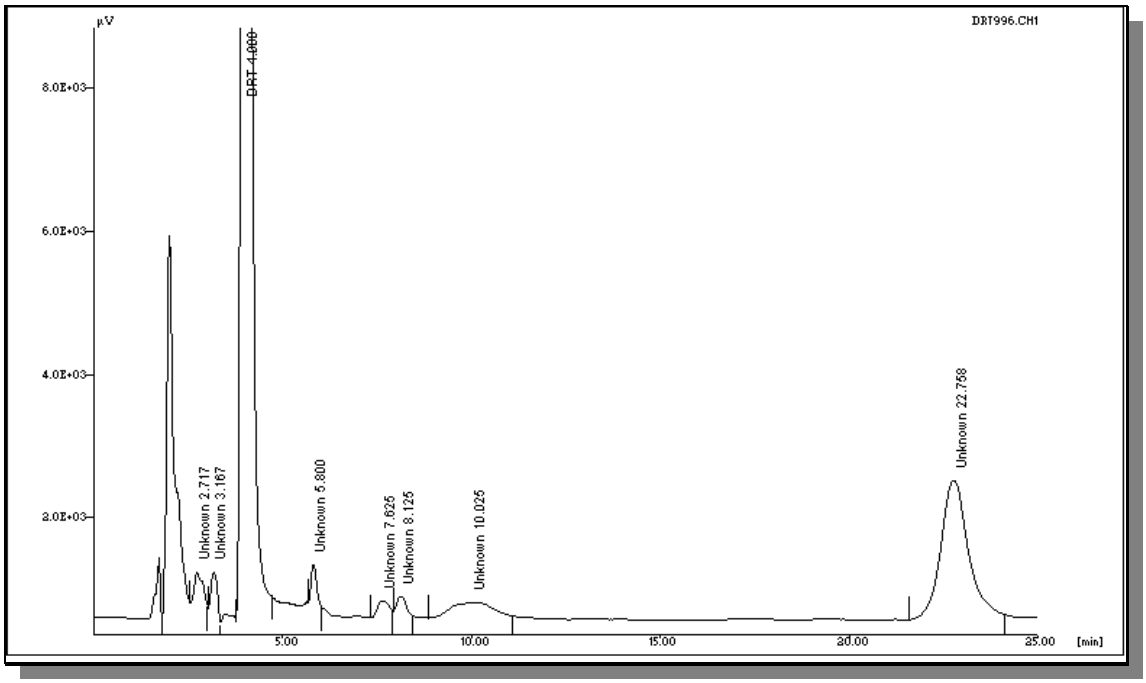
Chromatogram 9: Formulation A base degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.7	7385	1.4	679	1.09	-
2	Unknown	3.2	3281	1.15	2291	0.48	1.34
3	DRT	4	480159	1.26	2387	88.92	2.81
4	Unknown	6	13682	1.01	3127	2.02	5.26
5	Unknown	7.7	2782	0.99	6013	0.41	4.05
6	Unknown	8.1	1229	1.35	9712	0.18	1.22
7	Unknown	10.8	1026	1.09	7926	0.15	6.64
8	Unknown	13.9	1357	0.94	8683	0.2	5.63
9	Unknown	22.7	44448	1.14	6082	6.55	10.11



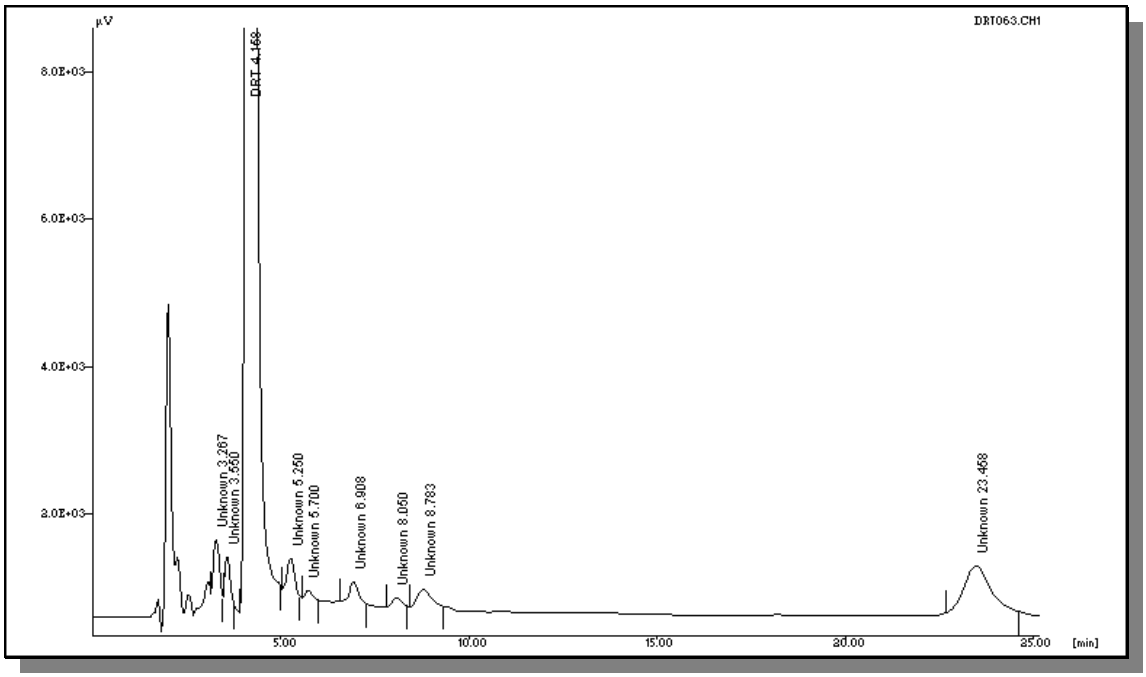
Chromatogram 10: Formulation B base degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.2	5936	1.17	2766	0.84	-
2	DRT	4	490664	1.33	2342	69.49	2.94
3	Unknown	6	9876	1.03	2259	1.4	4.81
4	Unknown	6.8	15249	1.19	5071	2.16	1.8
5	Unknown	7.7	3515	0.85	7855	0.5	2.35
6	Unknown	8.1	2520	1.26	10864	0.36	1.27
7	Unknown	11.4	4221	1.14	6813	0.6	7.6
8	Unknown	13.9	3188	1.12	6607	0.45	4.08
9	Unknown	15.7	8106	1.01	7357	1.15	2.52
10	Unknown	22.8	162849	1.06	6752	23.06	7.71



Chromatogram 11: Formulation C base degraded 10 µg/ml (zoomed)

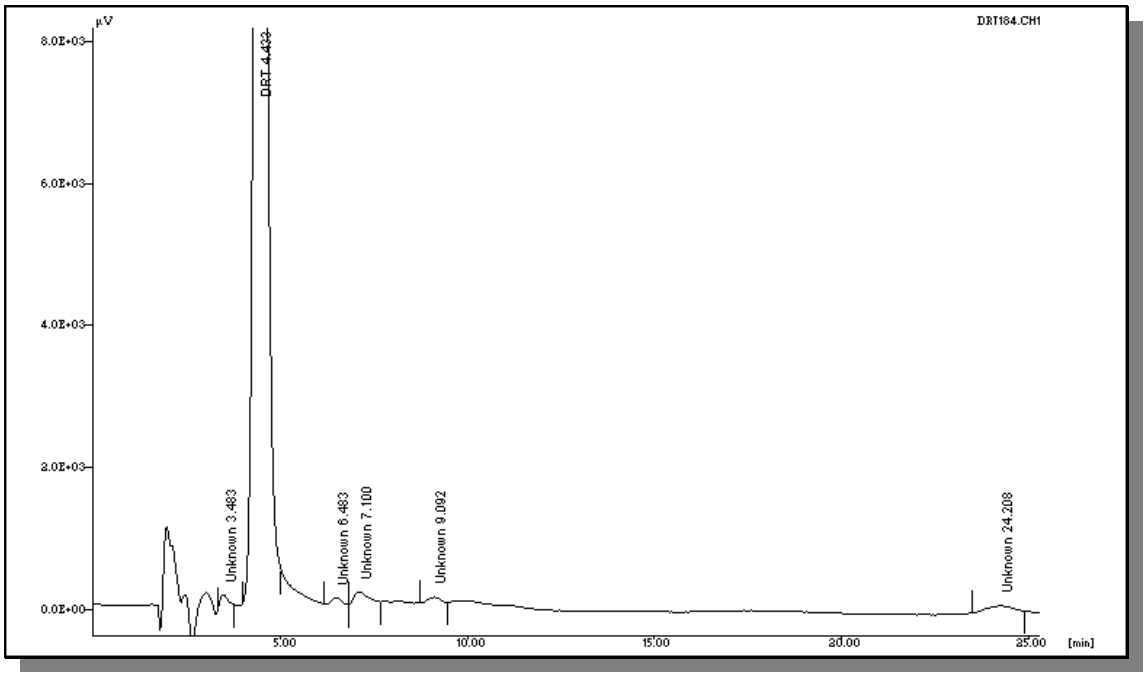
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.7	5864	1.48	770	1.08	-
2	Unknown	3.2	5499	1.14	2341	1.01	1.38
3	DRT	4	417204	1.34	2353	76.65	2.82
4	Unknown	5.8	4151	1.52	13009	0.76	6.77
5	Unknown	7.6	3003	1.04	4180	0.55	5.42
6	Unknown	8.1	3106	1.34	7853	0.57	1.2
7	Unknown	10	14696	0.96	408	2.7	1.62
8	Unknown	22.8	90803	1.14	6136	16.68	8.11



Chromatogram 12: Formulation D base degraded 10 µg/ml (zoomed)

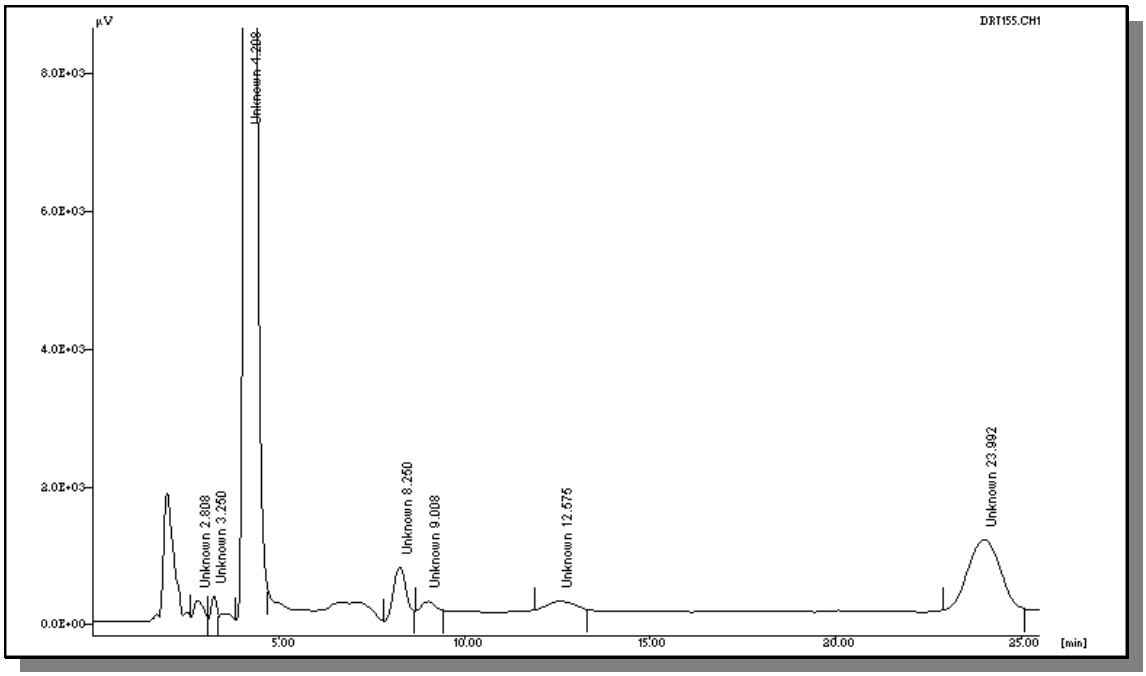
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.3	6231	1.2	3403	1.1	-
2	Unknown	3.6	4345	1.61	4794	0.77	1.32
3	DRT	4.2	510163	1.42	2358	89.93	2.23
4	Unknown	5.3	5351	1.14	4738	0.94	3.38
5	Unknown	5.7	1248	1.61	6748	0.22	1.55
6	Unknown	6.9	3769	1.33	6768	0.66	3.95
7	Unknown	8.1	1732	1.32	7124	0.31	3.19
8	Unknown	8.8	5122	1.26	3332	0.9	1.48
9	Unknown	23.5	29309	1.22	6295	5.17	16.42

Chromatograms of ACID degraded samples



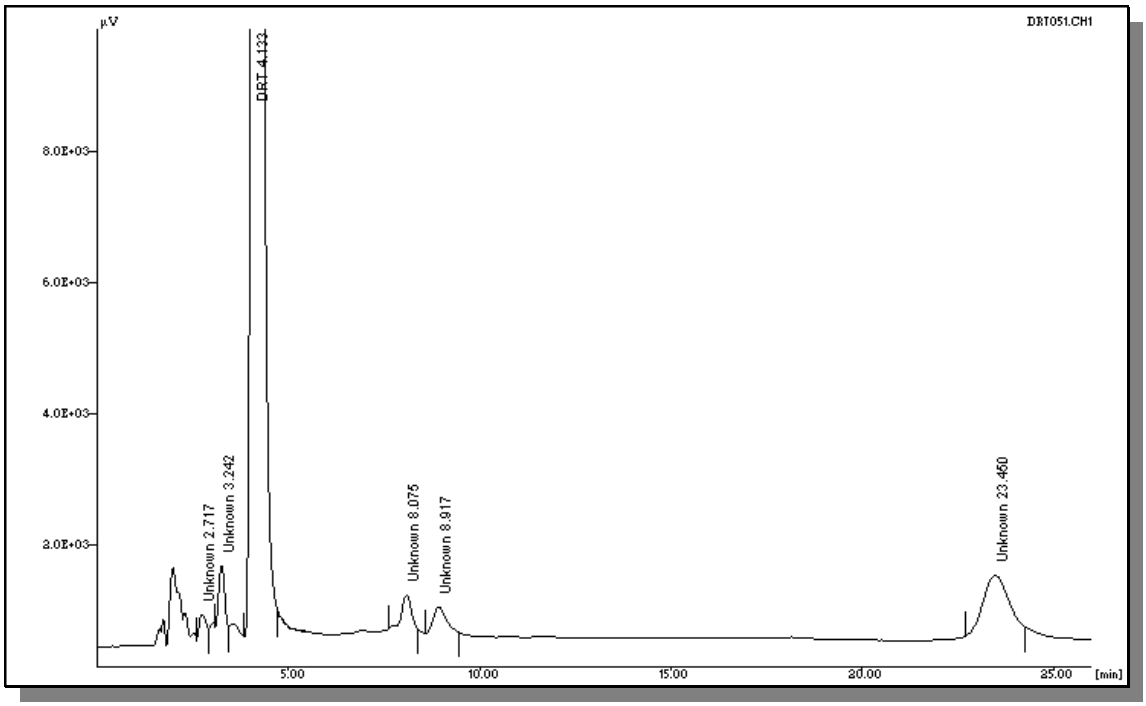
Chromatogram 13: API ACID degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.5	2341	1.37	1939	0.43	-
2	DRT	4.4	527749	1.18	1739	97.65	2.57
3	Unknown	6.5	1376	1.19	4169	0.25	4.97
4	Unknown	7.1	3566	1.58	2434	0.66	1.26
5	Unknown	9.1	1512	0.99	4195	0.28	3.51
6	Unknown	24.2	3892	0.95	5722	0.72	16.45



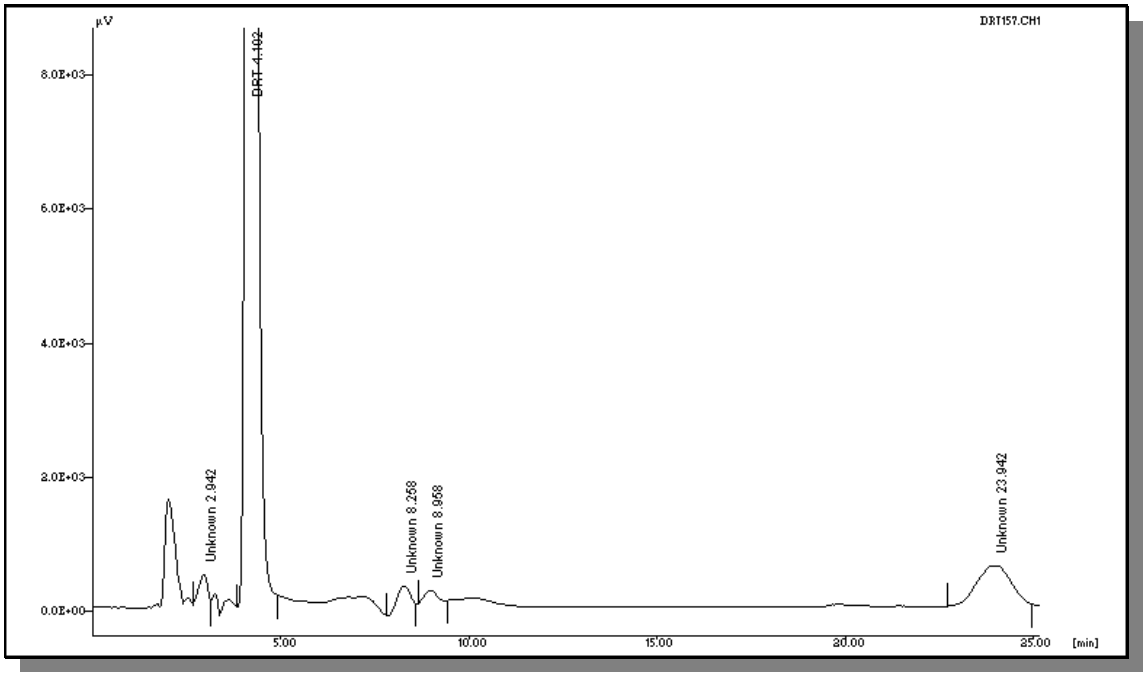
Chromatogram 14: Formulation A ACID degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.8	3490	1.46	786	0.61	-
2	Unknown	3.3	2260	0.95	3960	0.39	1.46
3	DRT	4.2	487756	1.19	1671	84.9	3.11
4	Unknown	8.3	13905	0.96	3833	2.42	8.57
5	Unknown	9	2915	1.18	3936	0.51	1.37
6	Unknown	12.6	5777	1.02	1399	1.01	3.73
7	Unknown	24	58405	0.97	3649	10.17	7.8



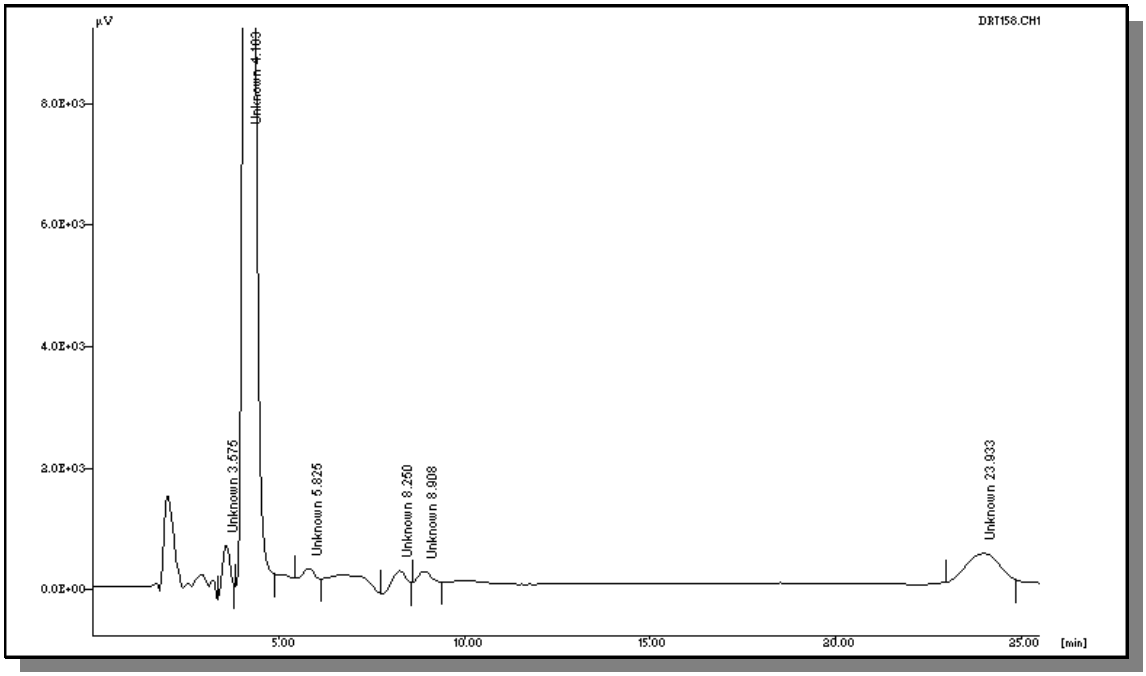
Chromatogram 15: Formulation B ACID degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.2	7510	1.1	3445	1.25	-
2	DRT	4.1	545537	1.4	1715	90.66	2.88
3	Unknown	8.1	7835	0.84	7720	1.3	10.3
4	Unknown	8.9	8722	1.4	3852	1.45	1.79
5	Unknown	23.5	32156	0.96	7184	5.34	17.33



Chromatogram 16: Formulation C ACID degraded 10 µg/ml (zoomed)

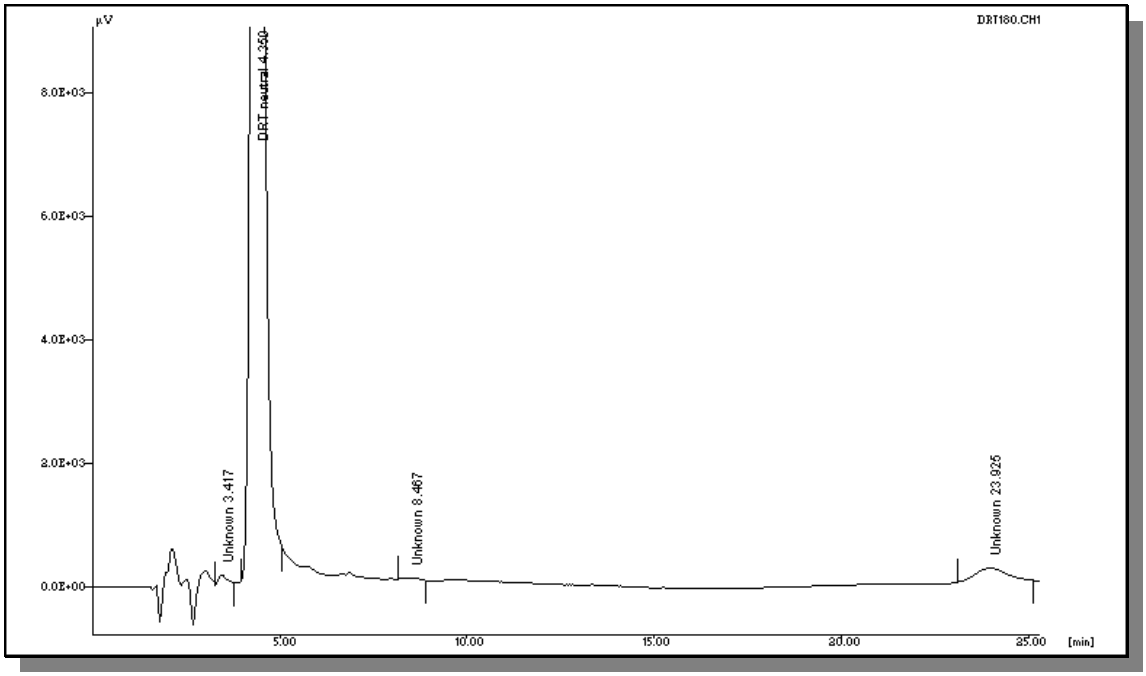
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.9	5916	0.81	828	1.11	-
2	DRT	4.2	482507	1.2	1564	90.24	3.01
3	Unknown	8.3	7103	0.91	3166	1.33	8.06
4	Unknown	9	3484	0.99	5525	0.65	1.31
5	Unknown	23.9	35658	0.94	3362	6.67	14.08



Chromatogram 17: Formulation D ACID degraded 10 µg/ml (zoomed)

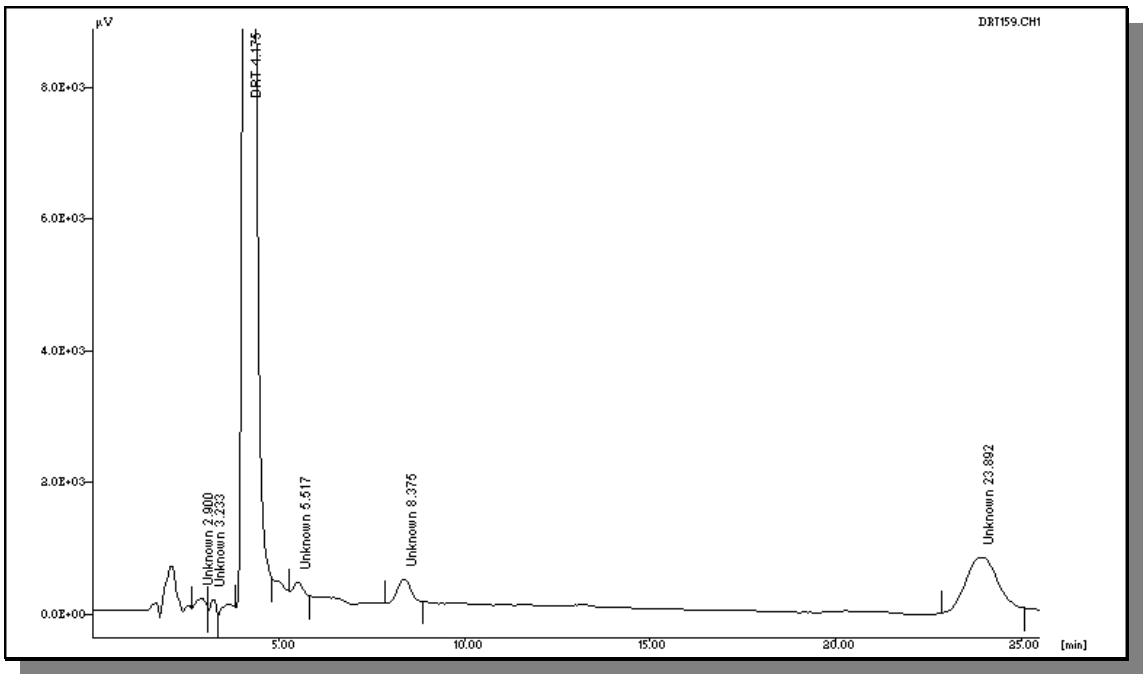
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.6	8761	1.01	2224	1.67	-
2	DRT	4.2	478940	1.2	1560	91.11	1.68
3	Unknown	5.8	2691	0.88	2810	0.51	3.81
4	Unknown	8.3	5436	0.82	3162	1.03	4.74
5	Unknown	8.9	3581	1.16	4761	0.68	1.2
6	Unknown	23.9	26280	0.97	3486	5	14.09

Chromatograms of Samples of Neutral degradation



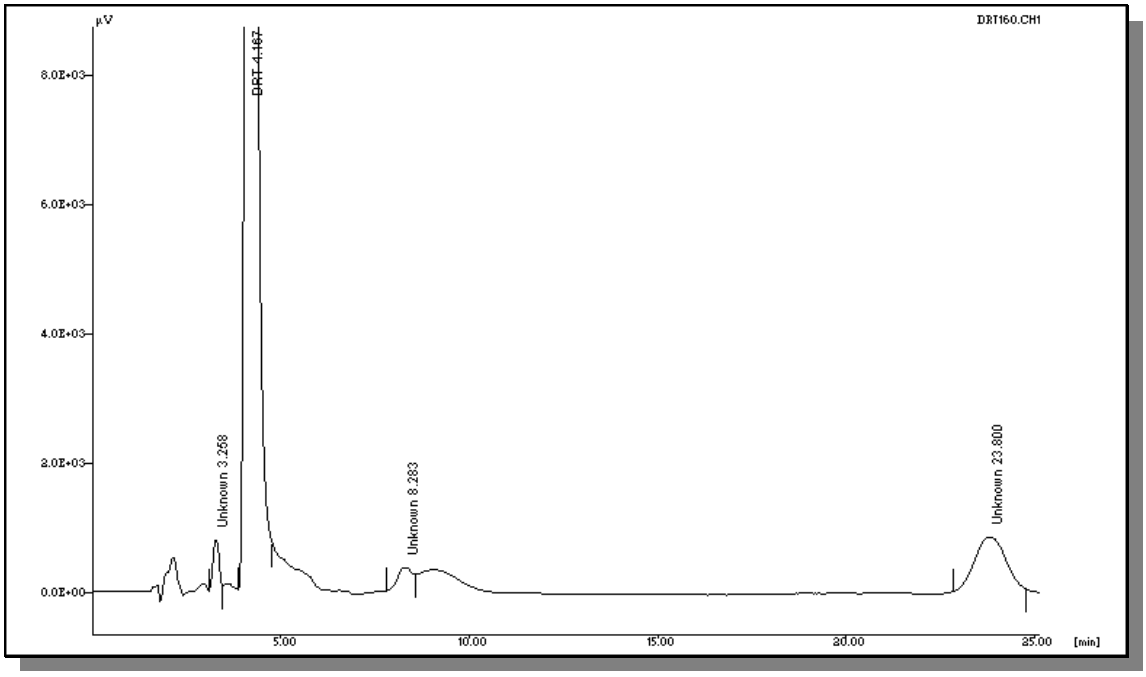
Chromatogram 18: API Neutral degradation 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.4	1998	1.87	1741	0.32	-
2	DRT	4.4	572347	1.26	1828	97.59	2.55
3	Unknown	8.5	967	1.26	2502	0.15	7.61
4	Unknown	23.9	12091	1.24	4068	1.94	14.23



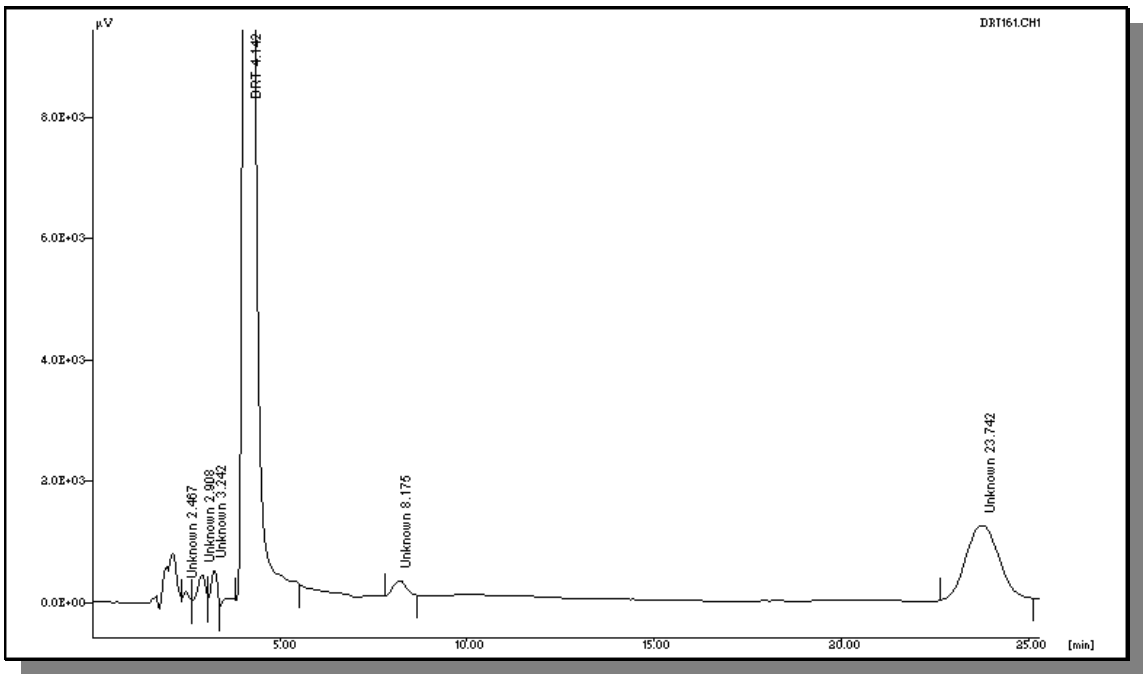
Chromatogram 19: Formulation A Neutral degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.9	2526	0.92	673	0.39	-
2	Unknown	3.2	1641	1.07	4342	0.26	1
3	DRT	4.2	567688	1.29	1950	90.68	3.3
4	Unknown	5.5	2297	1.26	3906	0.36	3.7
5	Unknown	8.4	7762	1.02	3376	1.21	6.2
6	Unknown	23.9	45404	1.05	4117	7.09	15.1



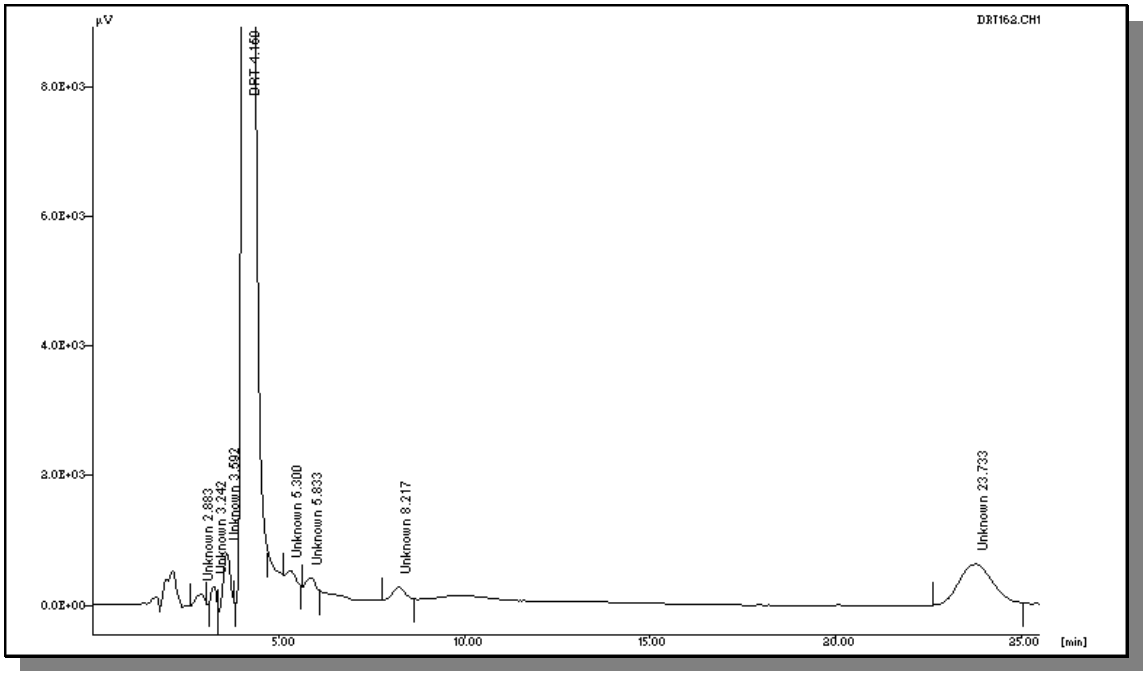
Chromatogram 20: Formulation B Neutral degraded 10 µg/ml (zoomed)

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.3	6569	0.95	3032	1.02	-
2	DRT	4.2	578689	1.3	1921	91.42	2.95
3	Unknown	8.3	3780	0.78	3754	0.59	8.96
4	Unknown	23.8	44638	1.01	4148	6.96	15.4



Chromatogram 21: Formulation C Neutral degraded 10 µg/ml (zoomed)

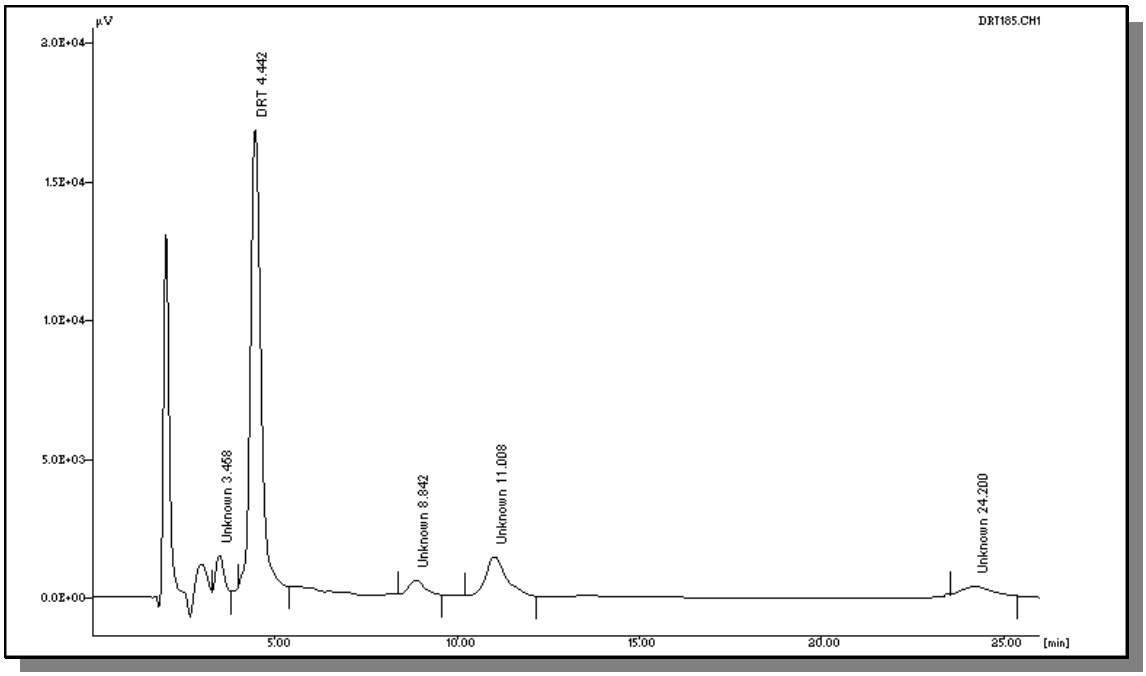
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.5	1227	1.49	2255	0.21	-
2	Unknown	2.9	4531	0.84	1474	0.77	1.7
3	Unknown	3.2	4631	0.95	2856	0.79	1.2
4	DRT	4.1	499986	1.31	1847	85.14	2.9
5	Unknown	8.2	5359	1.1	3330	0.91	8.5
6	Unknown	23.7	71531	1.05	3561	12.18	14.5



Chromatogram 22: Formulation D Neutral degraded 10 µg/ml (zoomed)

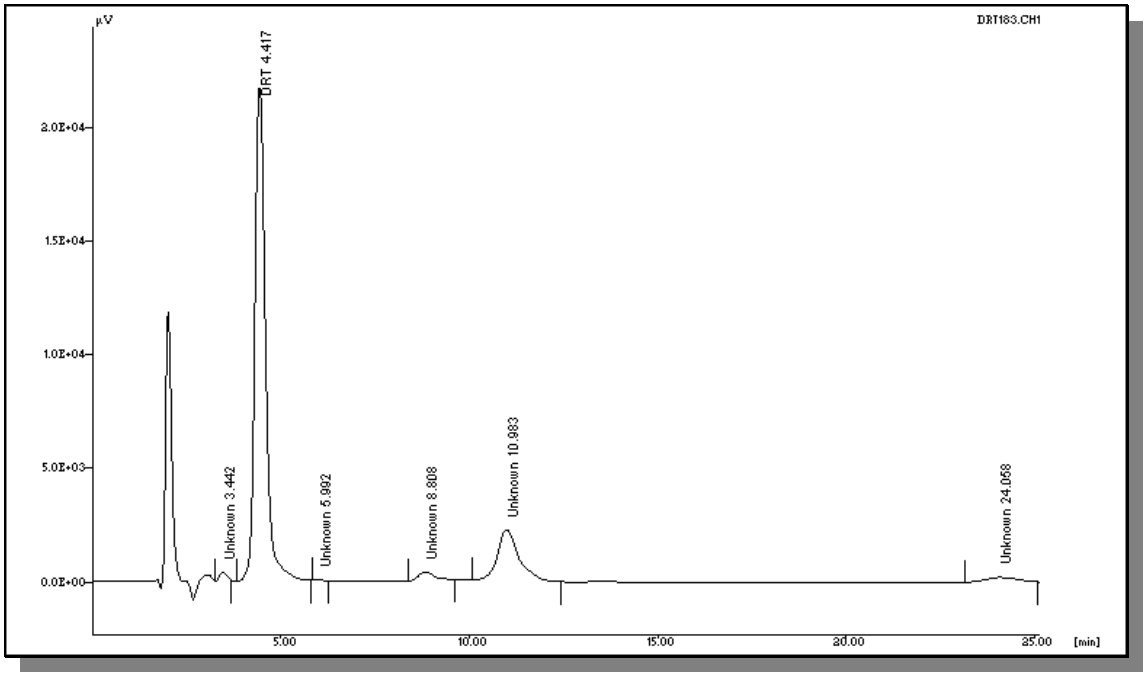
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.9	2242	0.87	886	0.33	-
2	Unknown	3.2	2866	1.07	3318	0.42	1.2
3	Unknown	3.6	9030	1.04	2687	1.33	1.4
4	DRT	4.2	547887	1.33	1869	91.28	1.7
5	Unknown	5.3	1599	1.54	3199	0.24	3
6	Unknown	5.8	2278	1.28	3669	0.34	1.4
7	Unknown	8.2	3903	1.04	3418	0.58	5
8	Unknown	23.7	37097	1.07	3461	5.48	14.3

Chromatograms of Chemical Oxidation



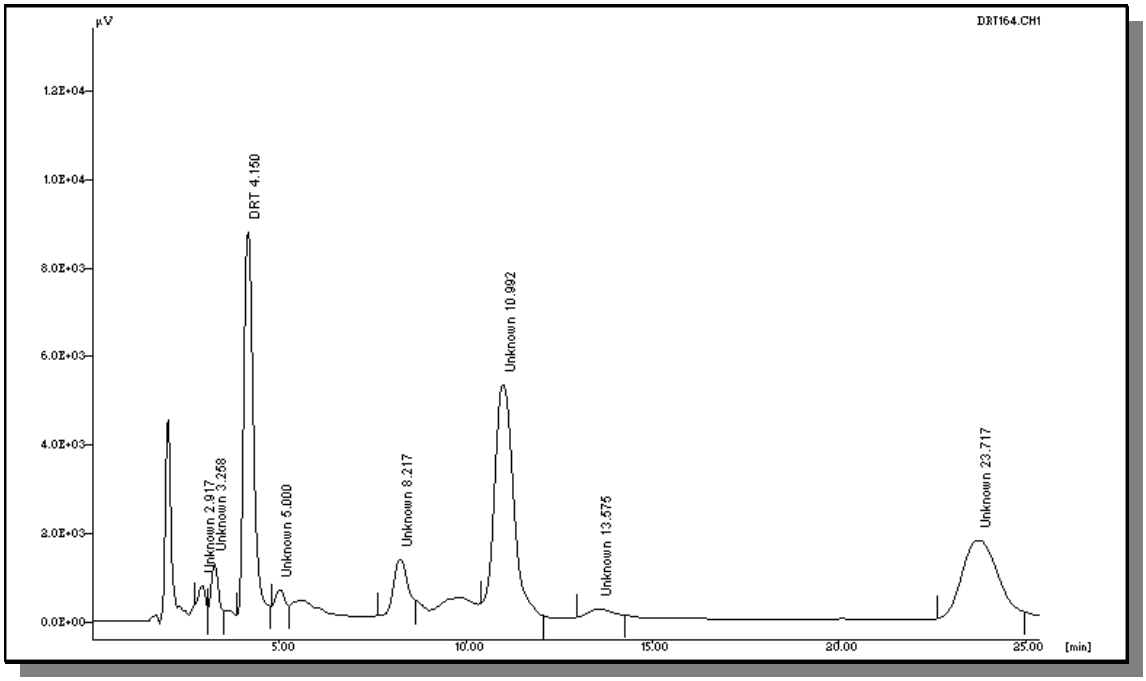
Chromatogram 23: API 10 µg/ml chemical oxidation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.5	16200	1.29	1754	4.26	-
2	DRT	4.4	282979	1.22	1832	74.35	2.64
3	Unknown	8.8	13664	1.35	2800	3.59	8.14
4	Unknown	11	51849	1.31	2669	13.62	2.86
5	Unknown	24.2	15898	1.3	4500	4.18	11.52



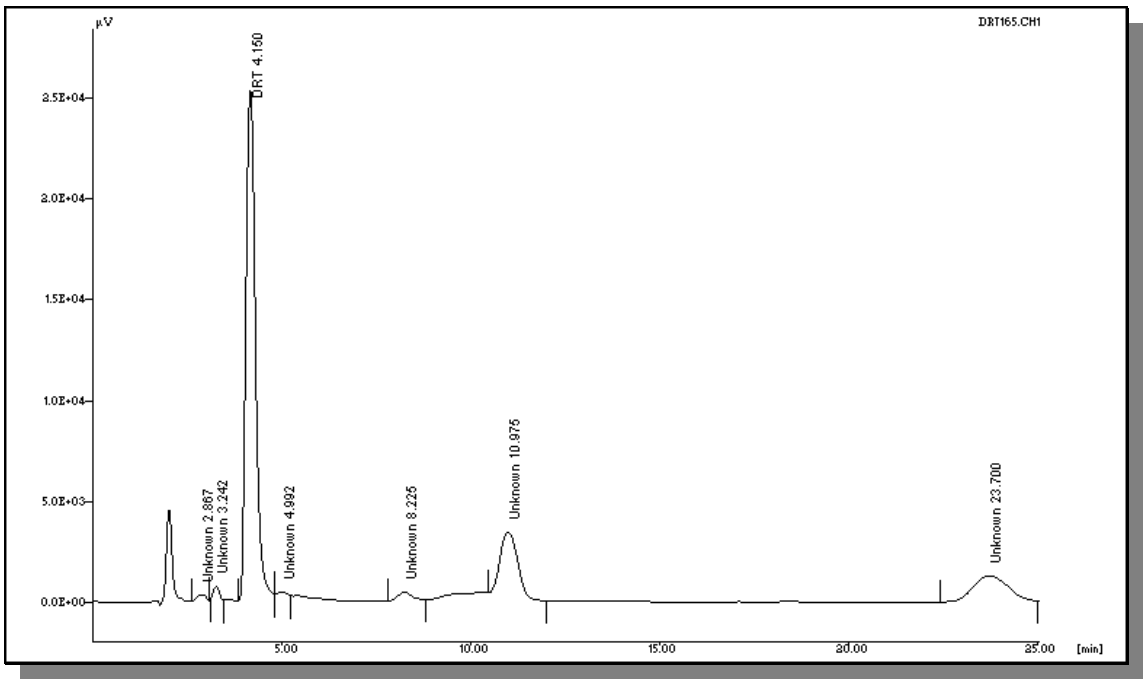
Chromatogram 24: Formulation A 10 µg/ml chemical oxidation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.4	4611	1.24	1809	0.95	-
2	DRT	4.4	376985	1.27	1778	77.41	2.63
3	Unknown	6	559	1.28	5466	0.11	4.25
4	Unknown	8.8	10150	1.44	2744	2.08	5.66
5	Unknown	11	82873	1.3	2663	17.02	2.86
6	Unknown	24.1	11847	1.07	4186	2.43	11.21



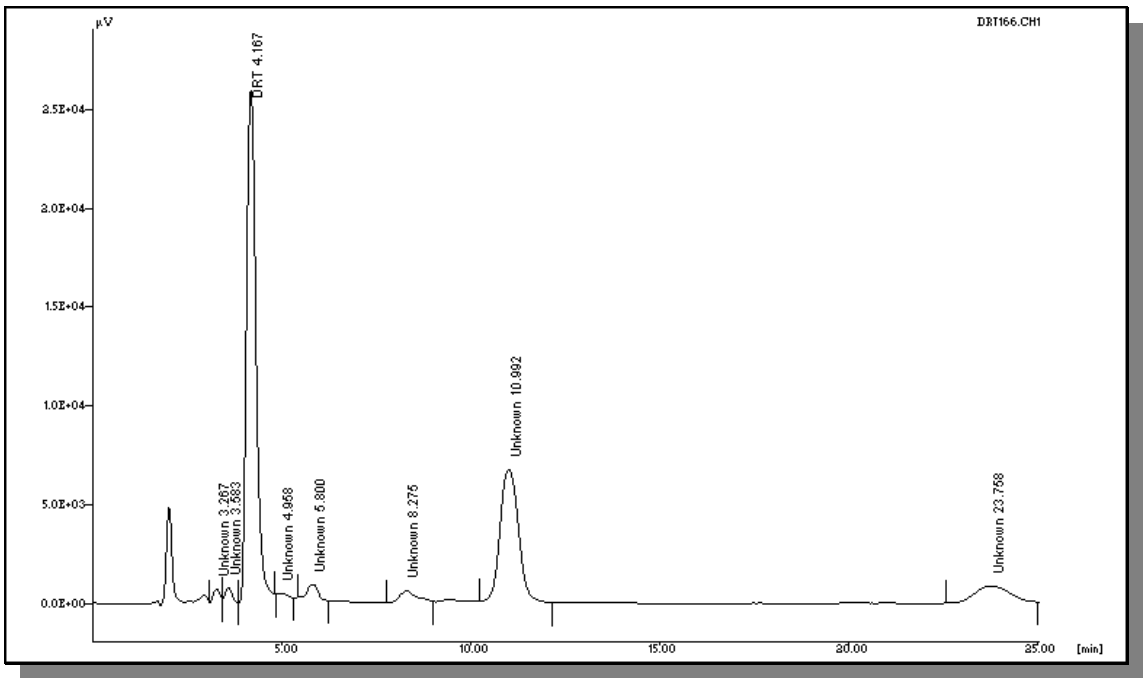
Chromatogram 25: Formulation B 10 µg/ml chemical oxidation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.9	4810	0.92	1883	1.09	-
2	Unknown	3.3	10447	1.1	2407	2.37	1.3
3	DRT	4.2	129760	1.27	1882	29.45	2.8
4	Unknown	5	4495	1.02	3514	1.02	2.4
5	Unknown	8.2	21870	1.01	3536	4.96	7.2
6	Unknown	11	158897	1.35	2875	36.06	4.1
7	Unknown	13.6	5840	1.06	2958	1.33	2.8
8	Unknown	23.7	104478	1.1	3213	23.71	7.6



Chromatogram 26: Formulation C 10 µg/ml chemical oxidation

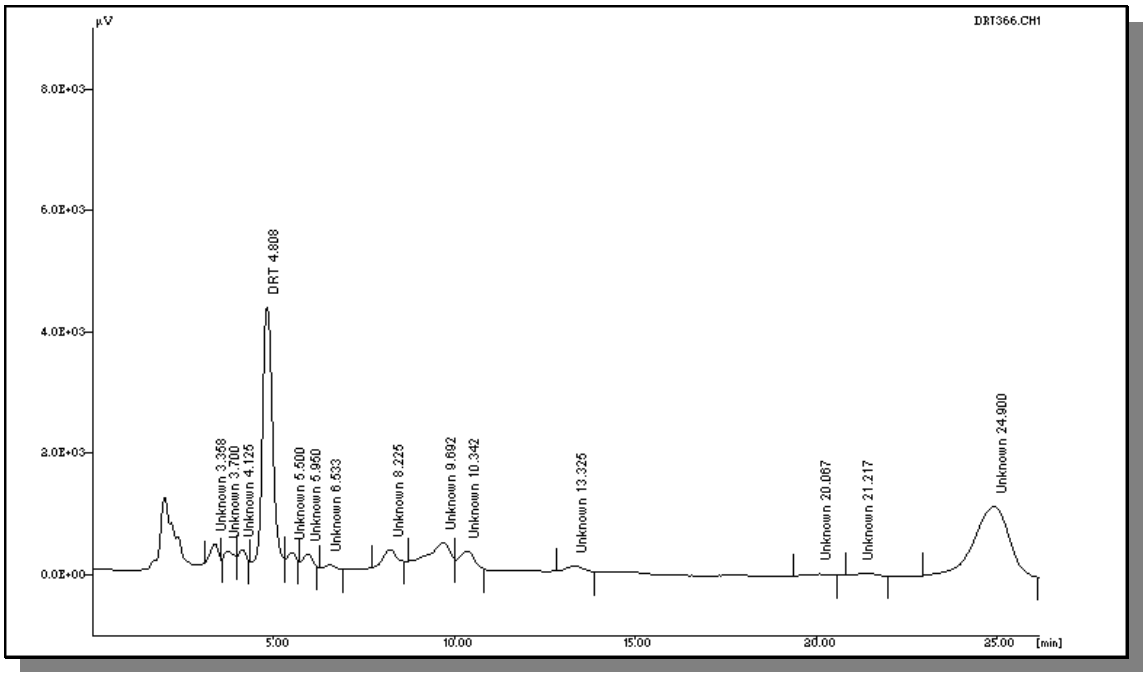
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.9	3708	0.82	730	0.65	-
2	Unknown	3.2	5997	1.06	2756	1.05	1.1
3	DRT	4.2	381123	1.31	1796	66.83	2.9
4	Unknown	5	1774	1.22	3541	0.31	2.3
5	Unknown	8.2	8581	1.12	3103	1.5	7
6	Unknown	11	91436	1.21	2885	16.03	3.9
7	Unknown	23.7	77697	1.07	3131	13.62	10.2



Chromatogram 27: Formulation D 10 µg/ml chemical oxidation

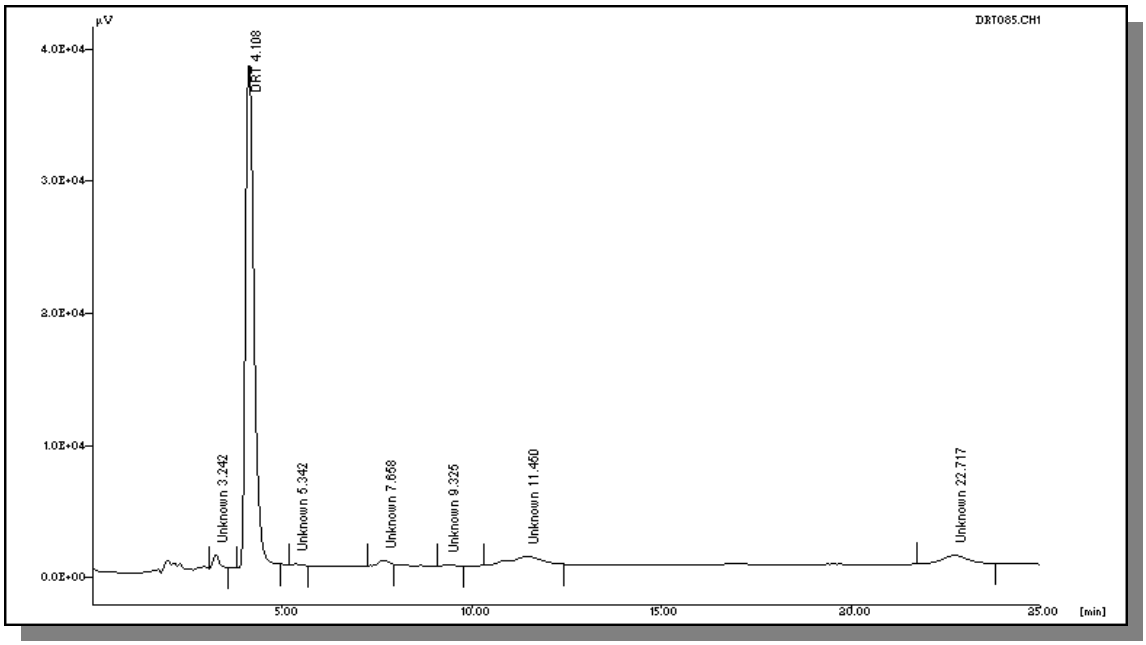
Sr No	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.3	5448	0.99	2671	0.79	-
2	Unknown	3.6	6057	1.25	2960	0.88	1.23
3	DRT	4.2	387167	1.27	1894	56.31	1.81
4	Unknown	5	1447	1.88	3157	0.21	2.16
5	Unknown	5.8	11692	1.25	2924	1.7	2.16
6	Unknown	8.3	15293	1.51	2454	2.22	4.52
7	Unknown	11	209626	1.12	2848	30.49	3.65
8	Unknown	23.8	50885	1.05	3220	7.4	10.24

Chromatograms of samples subjected to Photo degradation



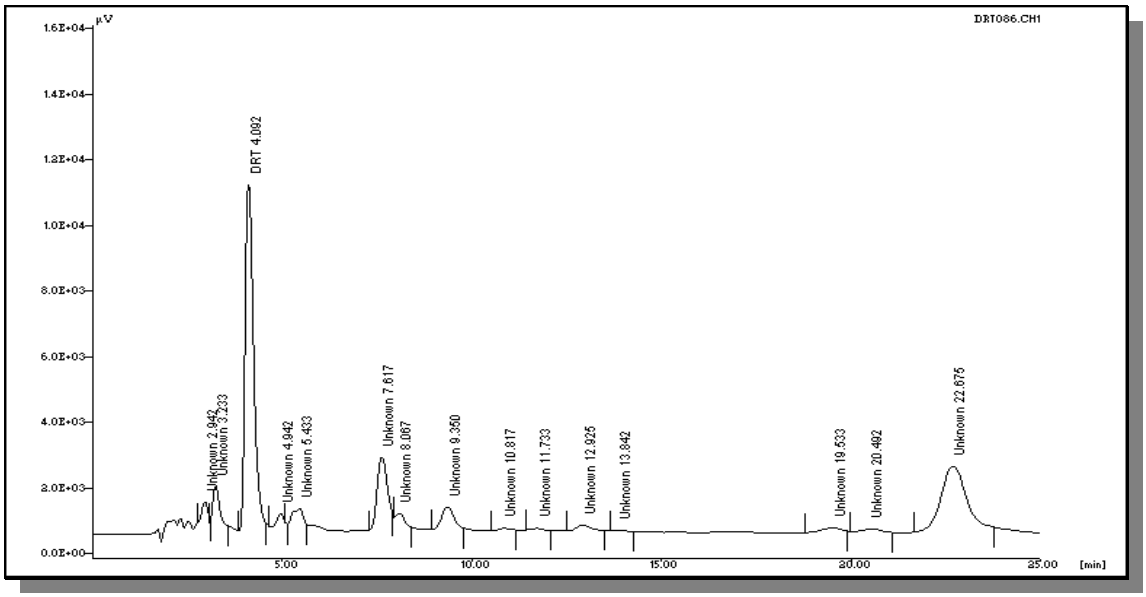
Chromatogram 28: API (10 µg/ml) subjected to Photo degradation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.4	3522	0.81	1578	1.99	-
2	Unknown	3.7	1572	1.43	1563	0.89	1
3	Unknown	4.1	1499	1.12	3515	0.85	1.31
4	DRT	4.8	67586	1.15	2112	38.25	1.97
5	Unknown	5.5	1469	1.03	4920	0.83	1.89
6	Unknown	6	2307	1.06	3904	1.31	1.3
7	Unknown	6.5	954	1.29	3055	0.54	1.37
8	Unknown	8.2	4791	0.84	3714	2.71	3.35
9	Unknown	9.7	8790	0.65	2967	4.97	2.35
10	Unknown	10.3	4415	1.27	4424	2.5	0.98
11	Unknown	13.3	2670	0.97	3971	1.51	4.07
12	Unknown	20.1	1027	0.87	5056	0.58	6.84
13	Unknown	21.2	1415	1.48	5985	0.8	1.04
14	Unknown	24.9	74686	0.82	3653	42.27	2.69



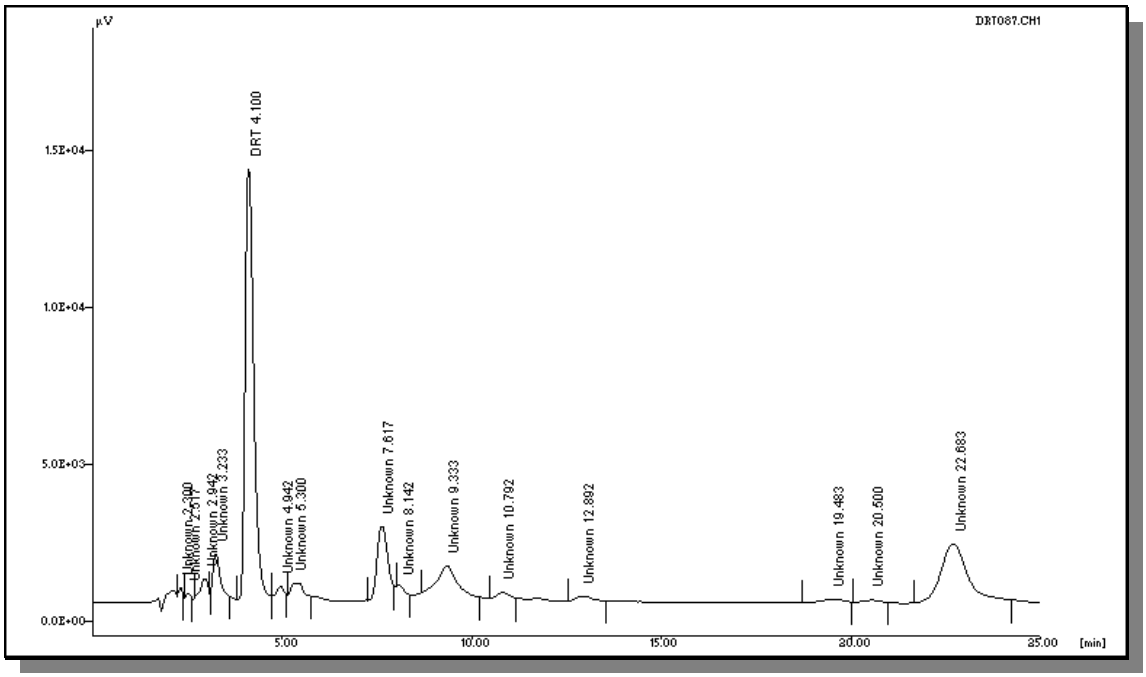
Chromatogram 29: Formulation A (10 µg/ml) subjected to Photo degradation

Sr No	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.2	9038	1.37	3344	1.27	-
2	DRT	4.1	510743	1.52	2382	71.77	3
3	Unknown	5.3	1090	1.53	2678	0.15	3.2
4	Unknown	7.6	5640	0.99	5216	0.79	5.5
5	Unknown	9.3	738	1.3	5072	0.1	3.5
6	Unknown	11.4	156308	0.93	685	21.96	1.8
7	Unknown	22.7	28079	1.01	6126	3.95	7.7



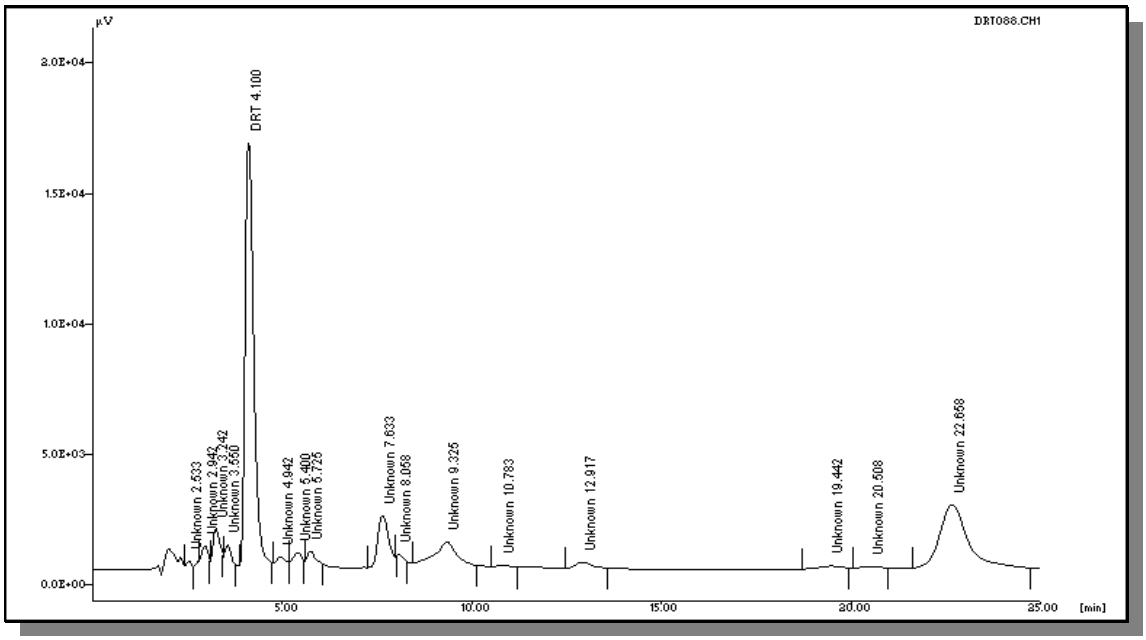
Chromatogram 30: Formulation B (10 µg/ml) subjected to Photo degradation

Sr No	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.9	5115	0.86	2591	1.67	-
2	Unknown	3.2	10686	1.55	3059	3.49	1.2
3	DRT	4	138387	1.52	2374	45.18	3
4	Unknown	4.9	3127	1.03	6164	1.02	2.8
5	Unknown	5.4	7420	0.86	2111	2.42	1.3
6	Unknown	7.6	30658	1.02	5180	10.01	4.8
7	Unknown	8	1995	2.01	9557	0.65	1.1
8	Unknown	9.3	12837	1.06	5747	4.19	3.1
9	Unknown	10.8	1350	1.06	5829	0.44	2.7
10	Unknown	11.7	918	0.98	10515	0.3	1.7
11	Unknown	12.9	4046	1.09	6676	1.32	2.1
12	Unknown	13.8	441	1.41	12578	0.14	1.6
13	Unknown	19.5	3779	0.75	7248	1.23	8
14	Unknown	20.4	2948	1.12	8723	0.96	1
15	Unknown	22.6	82602	1.07	6947	26.97	2.2



Chromatogram 31: Formulation C (10 µg/ml) subjected to Photo degradation

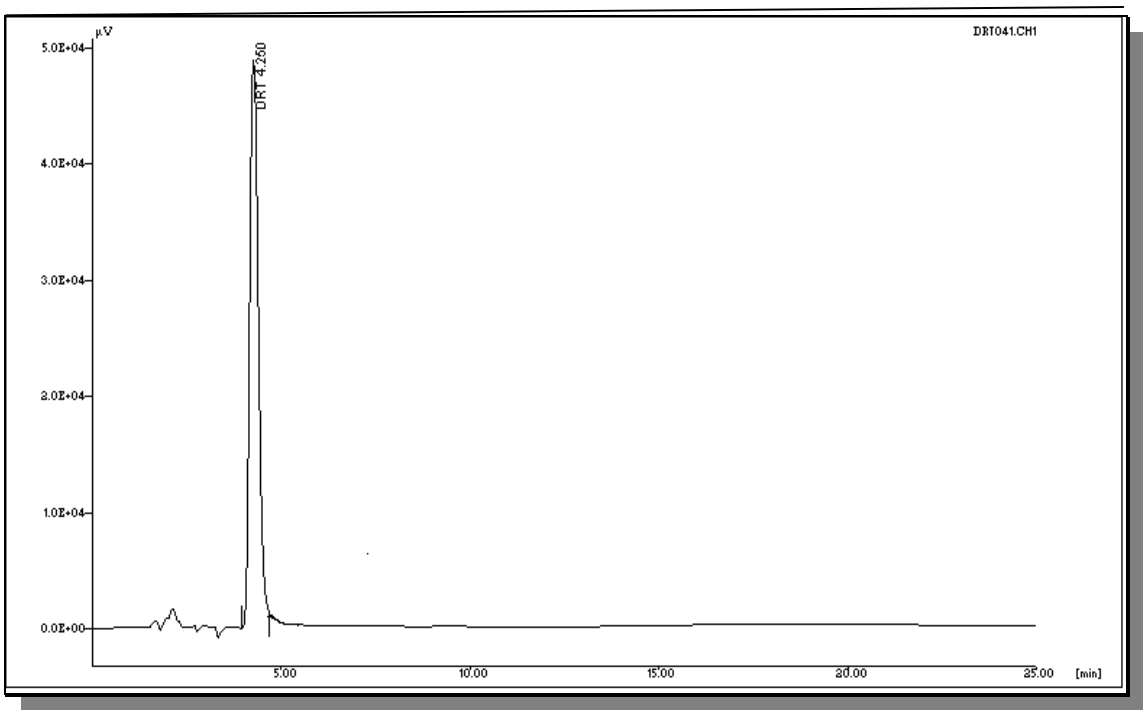
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.3	1380	1.13	5546	0.35	-
2	Unknown	2.5	933	1.15	3663	0.24	1.4
3	Unknown	2.9	4979	0.76	2451	1.27	2.1
4	Unknown	3.2	13048	1.7	2713	3.33	1.2
5	DRT	4.1	181808	1.48	2459	46.45	3
6	Unknown	4.9	2608	0.94	5622	0.67	2.8
7	Unknown	5.3	6888	1.73	1854	1.76	0.9
8	Unknown	7.6	33849	1.06	5113	8.65	5
9	Unknown	8.1	19834	2.84	52930	5.07	1.8
10	Unknown	9.3	29749	1.06	2399	7.6	2.6
11	Unknown	10.7	3546	1.17	6412	0.91	2.2
12	Unknown	12.8	3941	1.44	6197	1.01	3.5
13	Unknown	19.4	3661	0.83	7119	0.94	8.3
14	Unknown	20.5	1827	1.01	12818	0.47	1.2
15	Unknown	22.6	83327	1.2	6872	21.29	2.4



Chromatogram 32: Formulation D (10 µg/ml) subjected to Photo degradation

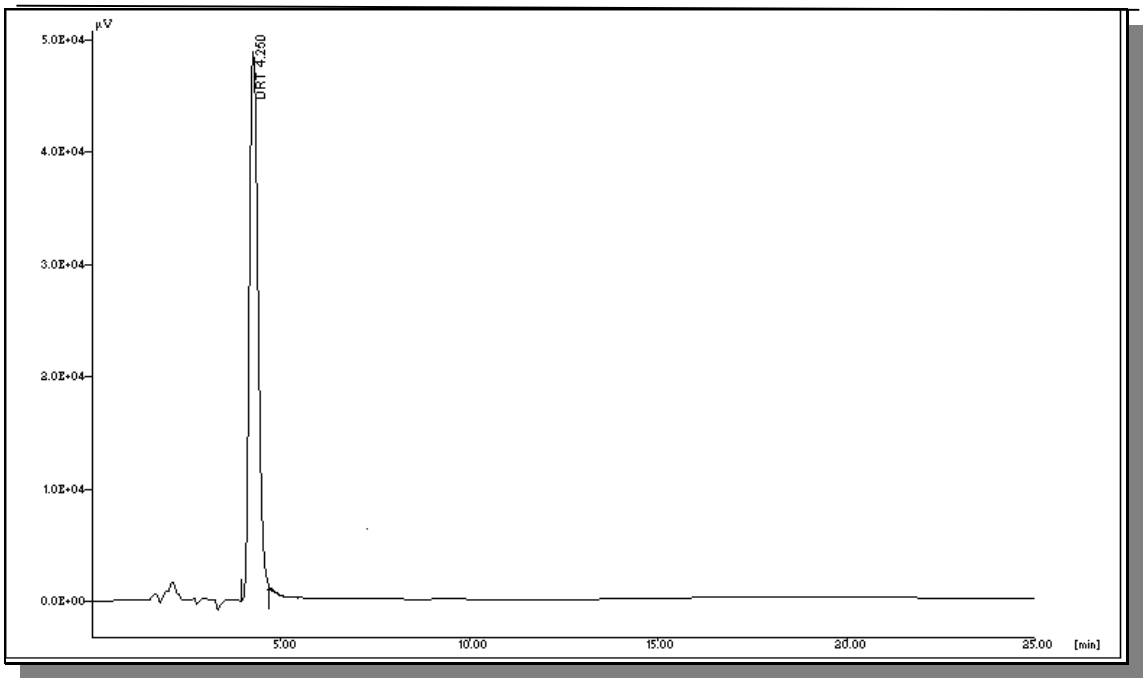
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	2.5	1262	0.99	3437	0.29	-
2	Unknown	2.9	4849	0.98	2451	1.11	1.9
3	Unknown	3.2	10611	1.23	2766	2.43	1.2
4	Unknown	3.5	3734	1.63	5278	0.85	1.3
5	DRT	4.1	215905	1.47	2425	49.43	2
6	Unknown	4.9	1997	1.19	5895	0.46	2.8
7	Unknown	5.4	3954	0.93	4082	0.91	1.5
8	Unknown	5.7	4675	1.74	7382	1.07	1
9	Unknown	7.6	27534	1.02	5355	6.3	5.5
10	Unknown	8	1291	1.81	17300	0.3	1.2
11	Unknown	9.3	30198	0.94	2110	6.91	2.4
12	Unknown	10.7	1472	1.24	6473	0.34	2.1
13	Unknown	12.9	6183	1.34	6031	1.42	3.5
14	Unknown	19.4	3130	0.84	5937	0.72	7.8
15	Unknown	20.5	2085	1.03	10483	0.48	1.1
16	Unknown	22.6	117922	1.44	6113	27	2.1

Chromatograms of samples of Thermolysis



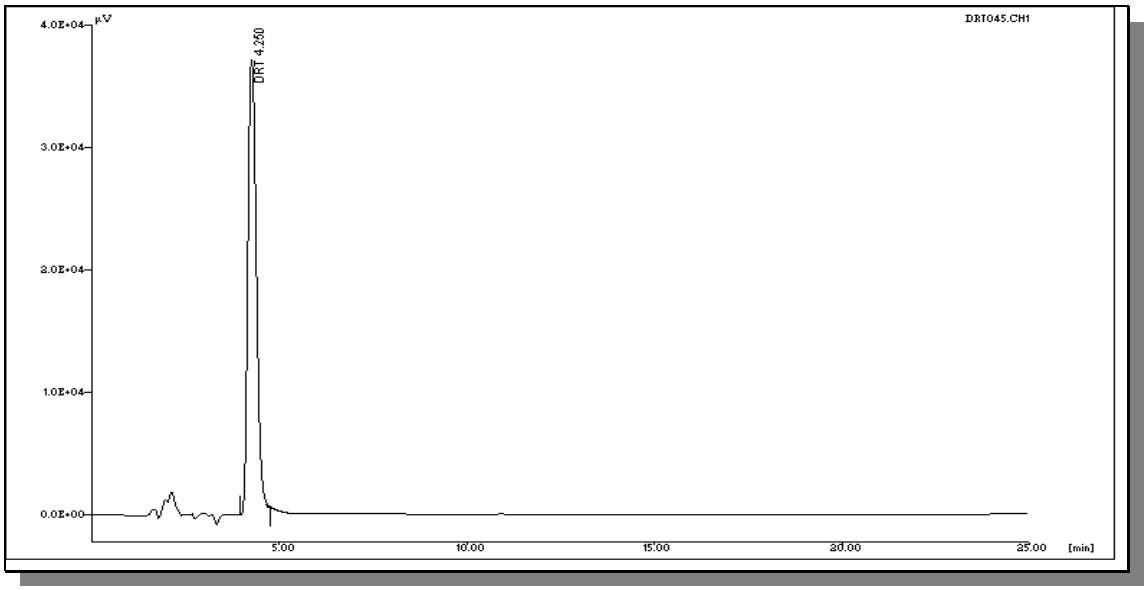
Chromatogram 33: API 10 µg/ml of thermal degradation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	4.2	608512	1.29	2337	100	-



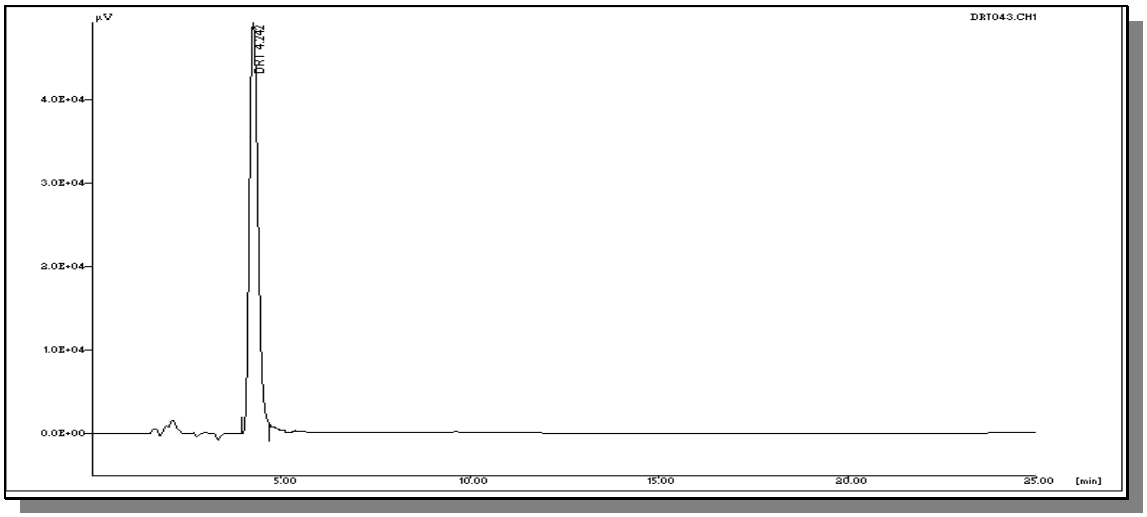
Chromatogram 34: Formulation A 10 µg/ml of thermal degradation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	4.2	655484	1.29	2337	100	-



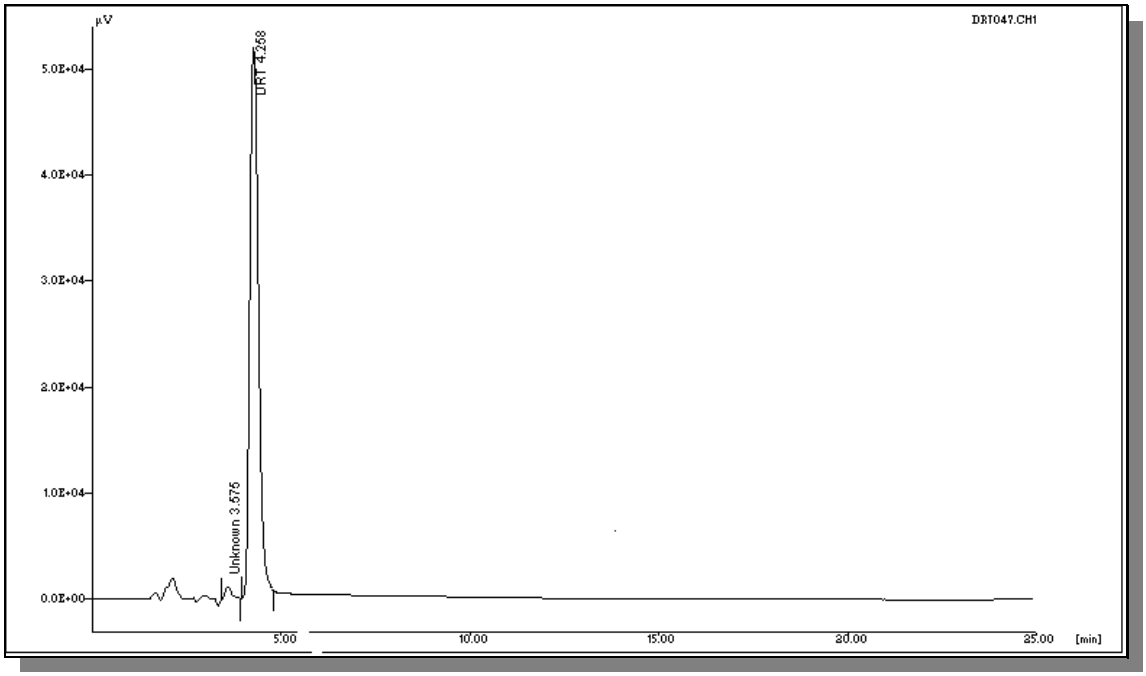
Chromatogram 35: Formulation B 10 µg/ml of thermal degradation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	4.2	596753	1.27	2357	98.8	-



Chromatogram 36: Formulation C 10 µg/ml of thermal degradation

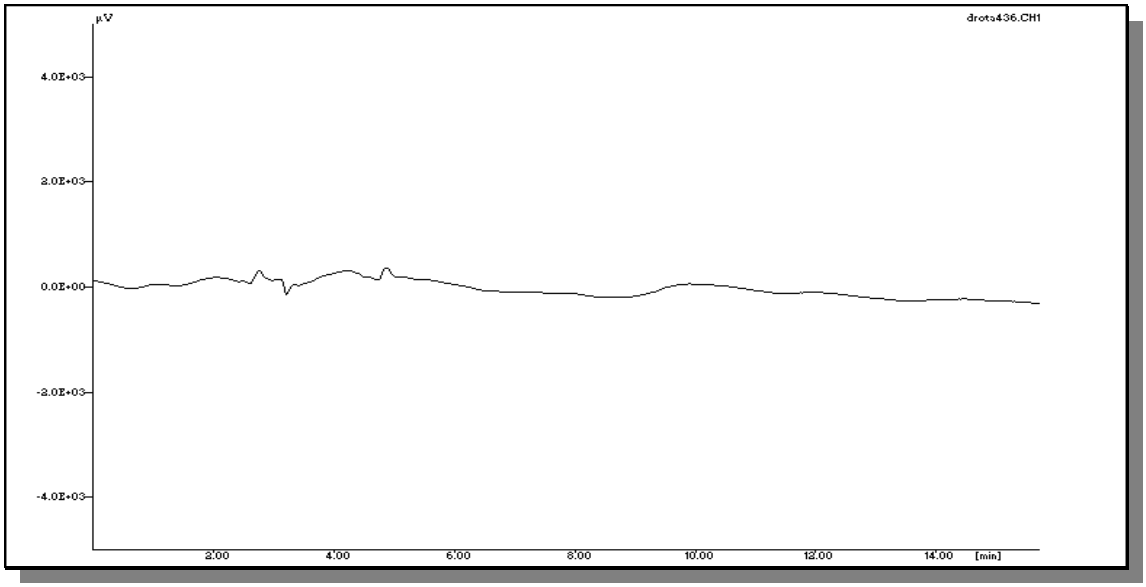
Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	DRT	4.2	602850	1.29	2394	100	-



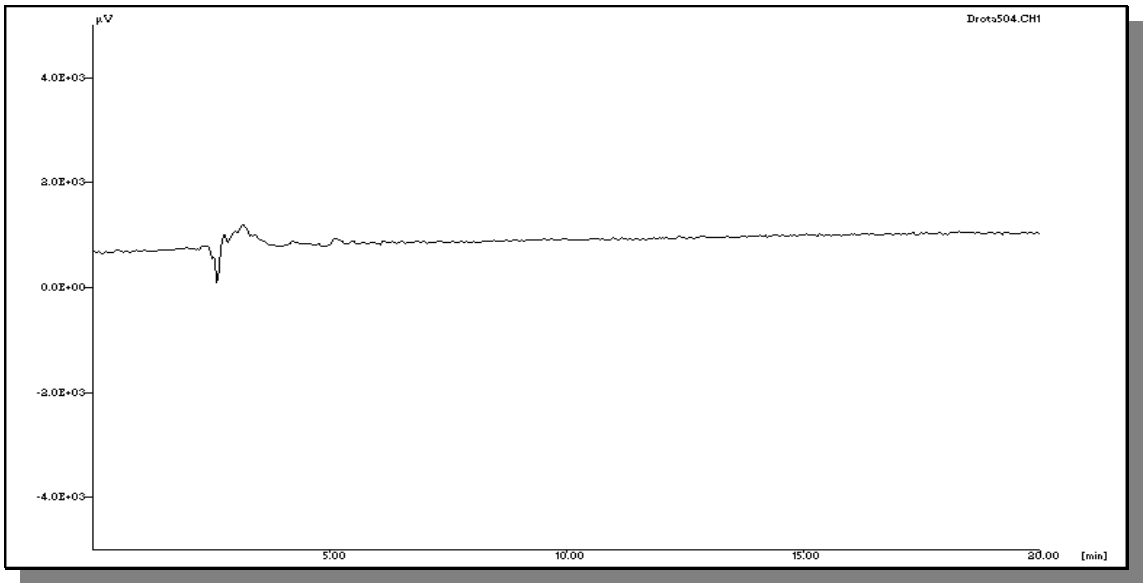
Chromatogram 37: Formulation D 10 µg/ml thermal degradation

Sr No.	Name	RT	Area[µV.Sec]	Asymmetry	Plates	%Area	Resolution
1	Unknown	3.5	12926	1.42	3386	1.79	-
2	DRT	4.2	607172	1.27	2401	96.67	2.3

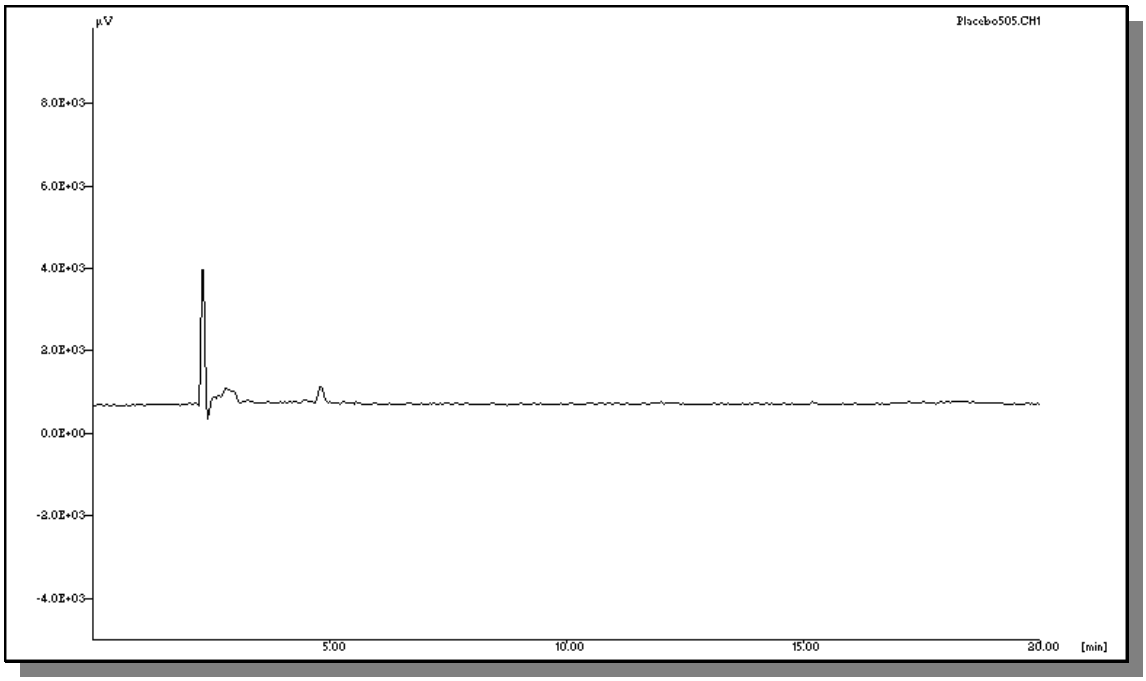
Placebo Degradation of Formulation A



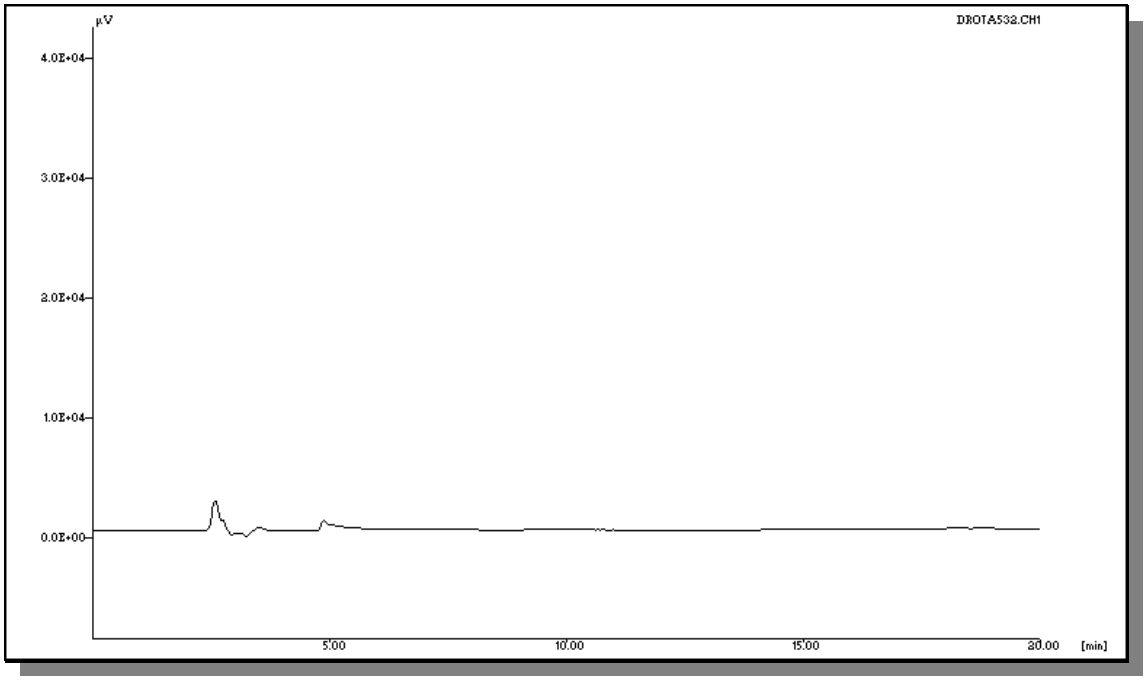
Chromatogram 38: Blank (ACN:Buffer)



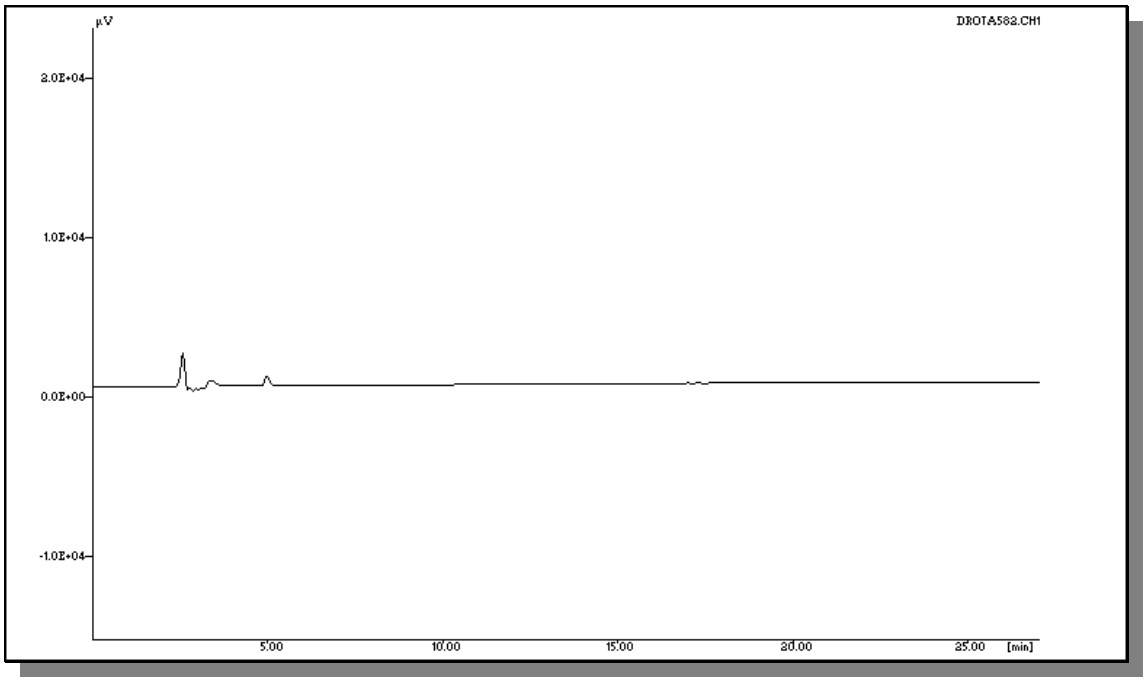
Chromatogram 39: Base Degraded Placebo



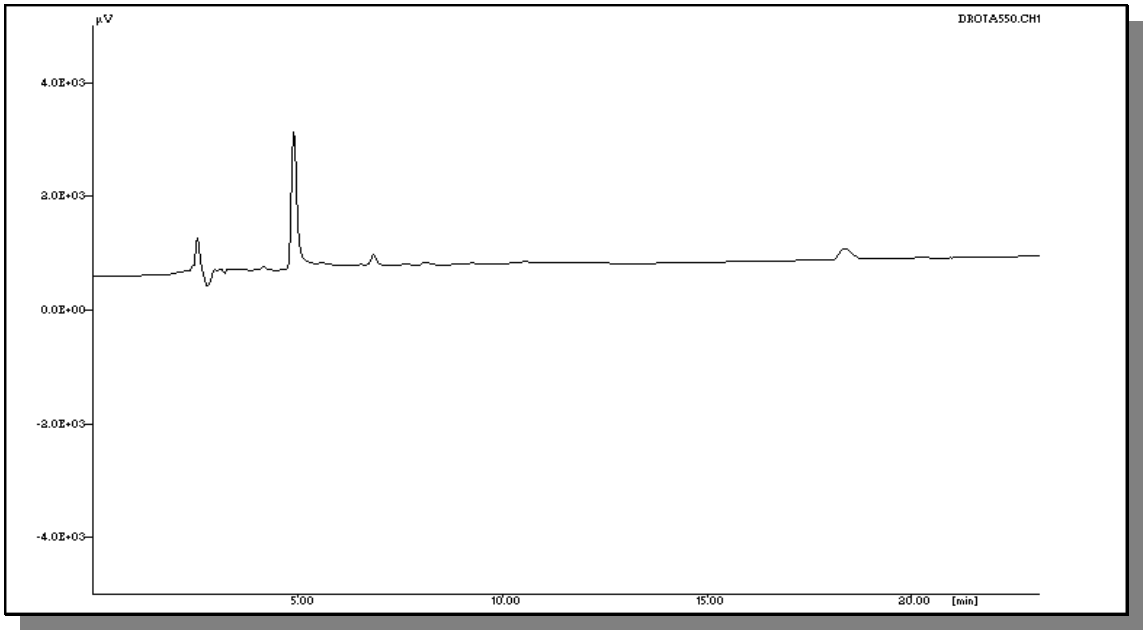
Chromatogram 40: Acid Degraded Placebo



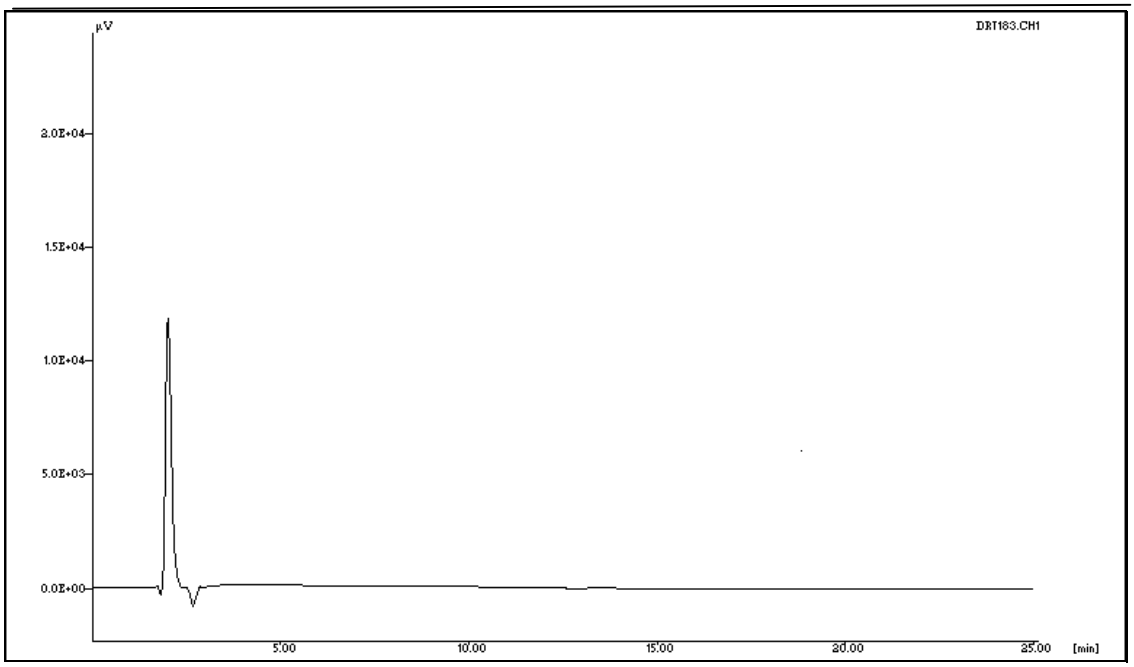
Chromatogram 41: Thermal Degraded Placebo



Chromatogram 42: Photo Degraded Placebo



Chromatogram 43: Neutral Degraded Placebo



Chromatogram 44: Peroxide Degraded Placebo

The optimization of mobile phase was carried out by taking the different proportion of organic and aqueous phase to obtain rapid simple assay method for Drotaverine HCl with appropriate run time, asymmetric factor and theoretical plate. Mobile phase consisting of Acetonitrile: 0.01M KH_2PO_4 buffer (43:57% v/v) & pH - 4 adjusted with dilute orthophophoric acid was found to be satisfactory which gave symmetric and sharp peak for DRT at 0.8 ml/min flow rate. UV spectrum of DRT showed peaks at 360, 302 & 240 nm. For quantitative analytical purpose 240 nm was set, which provided reproducibility with minimum interference than other UV bands. Under the chosen experimental condition, liquid chromatogram of DRT showed single peak at 4.2 min with asymmetric factor of 1.26.

The calibration curve was obtained by plotting the peak area versus concentration. It was found to be linear in the range of 0.5 -32 µg/ml. Peak area and concentration was subjected to least square regression and correlation coefficient. The data of calibration curve is shown in Table 17. The correlation coefficient was found to be 0.9999, showing good linearity.

The accuracy study was performed by standard addition method. The recovery of added standard to sample was calculated and found to be in range 98-102%, which indicated good accuracy of method (Table 21).

Precision study was carried out at 3 different concentration (5, 10, 15 µg/ml) and results of intraday and interday precision were reported in term of RSD (Table 18, 19, 20).Repeatability study was performed by six determination of working standard ie.10 µg/ml & result was reported in term of % RSD.

In specificity, study peak of DRT obtained without interference of degradation products.

LOD value of was found to be 0.08 µg/ml which is concentration that yields S/N ratio 3:1. LOQ value was found to be 0.24 µg/ml which is concentration that yields S/N ratio 10:1.

Robustness was performed by change in organic composition, flow-rate & pH of mobile phase; results were reported in terms of % recovery.

RSD value of DRT assay during solution stability study was within 2.

The proposed LC method applied to Formulation A, B, C & D. The results obtained were satisfactorily accurate precise.

Forced degradation was carried out as per ICH guidelines to both API and formulations. The objective of the study to find out the likely degradation products, which in turn help in establishing the degradation pathway and intrinsic stability of

molecule. In ordered to check selectivity of the method, degradation studies were carried out using hydrolysis (acid, base, neutral), thermolysis and chemical oxidation.

Upon heating the drug in presence of 1 N NaOH for 1 hr, degradation of drug was found to be 24.97 %. The peaks are well resolved. When DRT solution was subjected to 1N HCl for 4 hrs, degradation of the drug was found to be 16.67 %. For the wet degradation, degradation of the drug was found to be 4.49%. Chromatogram of API showed the % recovery 47.19% when subjected to 3 % H_2O_2 for 45 mins. No degradation observed in case of thermal degradation. Chromatogram of photo degradation was not fully resolved and showed recovery of 11.8%. There was no marked difference in chromatographic pattern as well as total degradation of DRT in all formulations compare to bulk drug under same conditions as shown in Table 26.

The proposed method, stability indicating assay method was applied to four different brands of injections of DRT for forced degradation study and determination of drug. The method was validated and found to be simple, sensitive accurate and precise. Statistical analysis proved that method was repeatable and selective for the analysis of DRT. The method was successfully applied to analysis of API in pharmaceutical formulations. Also the above method indicated the suitability of the method for acid, base, neutral, thermal and chemical oxidation study. As the method separated the drug from in degradation products, it can be used for analysis of stability samples.

Conclusion:

- DRT was found to be stable in thermal degradation & more susceptible to oxidation and photo degradation. DRT was found to be hydrolyzed in the acid & base, too.
- Method was found to be simple, precise, accurate & also specific as it did not show any interference from degradation products of DRT.
- There was no significant difference in the degradation of all formulations containing DRT HCl.
- The method is also applicable for the quantification of DRT HCl in routine analysis.

6.1. VALIDATION AND SYSTEM SUITABILITY TEST PARAMETERS

Table 16: SYSTEM SUITABILITY TEST PARAMETERS

System suitability parameters	Proposed method
Retention time ± SD	4.2 min ± 0.2
Asymmetry ± SD	1.26 ± 0.08
Theoretical plates	2512
Reproducibility (n=6)	585786.7 (µV.sec of 10 µg/ml)

6.2. Calibration data for Drotaverine HCl

Table 17: CALIBRATION CURVE

Concentration (µg / ml)	Peak area(µV.sec) Mean* ± SD	% RSD
0.5	29107 ± 325	1.1
1	57253 ± 310.5	0.5
2	111668 ± 2217.5	2.0
4	224384 ± 4935.5	1.86
8	441298 ± 7591	1.7
16	865378 ± 5544	0.6
32	1701586 ± 15260.5	0.9

* mean of triplet (n=3)

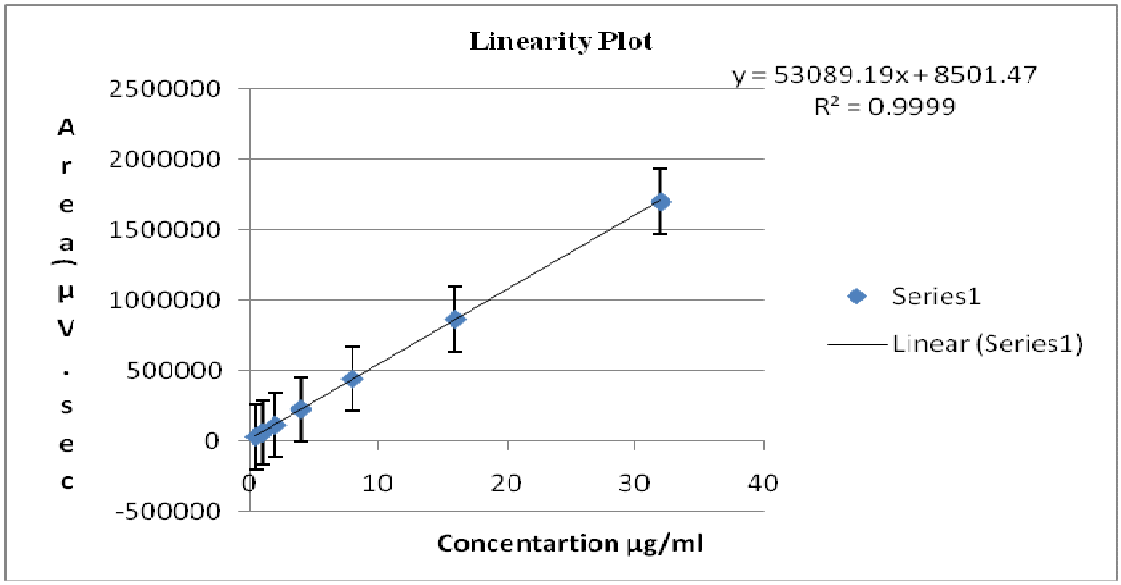


Figure 11: Linearity plot

6.3. Precision :

6.3.1. Intraday precision :

TABLE 18: INTRADAY PRECISION

Concentration (µg / ml)	Peak area (µV.sec)	Mean peak area(µV.sec)	SD	% RSD
5	298221	297284.3	855.88	0.28
	296543			
	297089			
10	588012	588099.7	691.67	0.11
	588831			
	587456			
15	882029	879109	2887.08	0.32
	879042			
	876256			

6.3.2 Interday precision :

TABLE 19: INTERDAY PRECISION

Concentration (µg / ml)	Peak area	Mean peak area (µV.sec)	SD	% RSD
5	298179	297220	867.76	0.29
	296489			
	296992			
10	591376	591323.7	456.75	0.07
	590843			
	591752			
15	881944	878779.3	3016.02	0.34
	878456			
	875938			

6.3.3 Repeatability :

TABLE 20: REPEATABILITY

Concentration (µg / ml)	Mean area* ± SD	%RSD
10	585786.7 ± 1355.3	0.23

* mean of triplet (n=6)

6.4. Recovery Study by Standard Addition Method:

TABLE 21: RECOVERY STUDY

working concentration (µg / ml)	Amount added (µg / ml)	Mean area observed*	Actual concentration (µg / ml)	Concentration recovered * (µg / ml)	%Recovery* ± SD
10	8	952600.3	18	18.35	101.94 ± 0.05
	10	1039674	20	20.02	100.11 ± 0.26
	12	1131751	22	21.79	99.06 ± 0.37

* mean of triplet (n=3)

6.5. Robustness

TABLE 22: ROBUSTNESS

Sr. No.	Parameter	Factor	Assay* ± SD
1	Change in organic Phase Ratio	-5%	98.35 ± 0.24
		0%	100.63 ± 0.11
		+5%	99.6 ± 0.3
2	Change in pH of buffer of mobile phase	-0.20%	98.62 ± 0.42
		0%	100.63 ± 0.11
		+0.20%	98.1 ± 0.25
3	Change in Flow	-10%	97.63 ± 0.56

	rate of the method	0%	100.63 ± 0.11
		10%	99.22 ± 0.42

*mean of triplet (n=3)

6.6. Solution stability

TABLE 23: SOLUTION STABILITY

Parameter	Sample (10 ppm)	Area * (µV.sec)	Assay*± SD	% RSD
Initial At zero time	API	599642	-	-
	Formulation A	586976	97.9 ± 0.25	0.25
	Formulation B	592568	98.8 ± 0.25	0.25
	Formulation C	581270	96.93 ± 0.11	0.11
	Formulation D	596086	99.4 ± 0.2	0.20
Fresh API		603420	100.63 ± 0.11	0.11
After 24 hrs	API	592916	98.25 ± 0.07	0.07
	Formulation A	585786	97.07 ± 0.04	0.04
	Formulation B	593486	98.25 ± 0.15	0.15
	Formulation C	582154	97.08± 0.16	0.16
	Formulation D	594887	98.58 ± 0.08	0.08

* mean of triplet (n=3)

6.7. Assay :

TABLE 24: ASSAY RESULTS

Formulation	Label claim(mg/ml)	Amount found*(mg/ml)	% recovery * ± SD (BY HPLC)	% recovery * ± SD (BY UV-VIS)
Formulation A	20	19.58	97.22 ± 0.03	98.36 ± 0.2
Formulation B	20	19.76	98.82 ± 0.25	101.63 ± 0.37
Formulation C	20	19.38	96.93 ± 0.11	98.36 ± 0.46
Formulation D	20	19.88	99.41 ± 0.30	101.96 ± 0.76

* mean of triplet (n=3)

6.8. Summary of validation parameters

TABLE 25: Summary of Validation Parameters

Parameters		DRT
Linearity		Y=53,089.19x + 8501.47 r^2=0.9999
Accuracy		98-102 %
Detection limit		0.08 (µg / ml)
Quantitation limit		0.24 (µg / ml)
Accuracy		98-102%
Precision	Repeatability (n=6)	0.11-0.28
	Intraday(n=3)	0.11-0.32
	Interday(n=3)	0.07-0.34
Robustness		98-102% Assay
Solution Stability		0.04-0.25% RSD

6.9. % Degradation of Drotaverine HCl

TABLE 26: % Degradation

Condition	Time	API	Formulation A	Formulation B	Formulation C	Formulation D
Acid	4 hrs at 100°C	16.69	16.91	9.03	16.99	19.66
Base	1 hrs at 100°C	24.97	18.8	17.2	28.22	14.42
Neutral	4 hrs 100°C	4.49	3.29	2.35	14.04	8.09
Thermal	6 hrs at 80 °C	3.7	No degradation			
Chemical oxidation	45 min 100°C	52.81	35.78	78.11	34.44	35.05
Photo	Sun-light 6 hrs	89.2	19.65	76.43	68.73	63.78

Comparison of Degradation products of API with that of Formulations by Rt(min):

Acid degradation products:

- API: **3.5**, 6.4, 7.1, **9**, **24.2**
- Formulation A: 2.8, **3.3**, **8.3**, **9**, 12.6, **24.2**
- Formulation B: 2.7, **3.2**, **8.1**, **8.9**, **23.5**
- Formulation C : **2.9**, **8.3**, **9**, **23.9**

- Formulation D: **3.6**, 5.8, **8.3**, **8.9**, **23.9**

Neutral degradation products:

- API: **3.4, 8.5, 23.9**
- Formulation A: 2.8, **3.2**, 5.5**, 8.4**, **23.9**
- Formulation B: **3.3**, **8.3**, **23.8**
- Formulation C : 2.5, 2.9, **3.2, 8.2**, **23.7**
- Formulation D: 2.9, **3.2**, 5.3, 5.8, **8.2**,**23.9**

Base degradation products:

- API: **3.4**, **6.5**, **8.2**, 8.7, **24**
- Formulation A: 2.7, **3.2**, **6**, **7.7**, **8.1**, 10.8, 13.9, **22.7**
- Formulation B: **3.2**, **6**, 6.8, **7.7**, **8.1**, **11.4**, 13.9, 15.7, **23.5**
- Formulation C : 2.7, 3.2, **5.8**, **7.6**, **8.3**, 10, **22.8**
- Formulation D: **3.3**, 3.6, 5.3, **5.7**, 6.9, **8.1**, 8.8, **23.5**

Oxidation products:

- API: **3.5**, **8.8**, **11**, **24.2**
- Formulation A: **3.4**, 4.2, **5.9**, **8.8**, **11**, **24.2**
- Formulation B: 2.9, **3.3**, **5**, **8.2**, **11**, 13.6, **23.7**
- Formulation C: 2.9, **3.2**, **5**, **8.2, 11**, **23.7**
- Formulation D: **3.3**, 3.6, **5**, 5.8, **8.2**, **11**, **23.8**

Photo degradation products:

- API: **3.4**, 3.7, **5.5**, 6, 6.5, 8.2, **9.7**, 10.3, 13.3, **20.1**, 21.2, **24.9**
- Formulation A: **3.2**, **5.3**, 7.6, **9.3**, 11.4, **22.7**
- Formulation B: 2.9, **3.2**, **4.9**, **5.4**, 7.6, **8**, **9.3**, **10.8**, 11.7, **12.9**, 13.8, **19.5**, **20.4**,**22.6**
- Formulation C : 2.3, 2.5, **3.2**, **4.9**, **5.3**, **8.1**, **9.3**, **10.7**, **12.8**, **19.4**, **20.5**, **22.7**
- Formulation D: 2.5, **2.9**, **4.9**, **5.4**, 5.7, 7.6, **8**, **9.3**, **10.7**, **12.9**, **19.4**, **20.5**, **22.6**

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